REMARKS

Claims 17-34 currently appear in this application. The Office Action of July 23, 2008, has been carefully studied. These claims define novel and unobvious subject matter under Sections 102 and 103 of 35 U.S.C., and therefore should be allowed. Applicant respectfully requests favorable reconsideration, entry of the present amendment, and formal allowance of the claims.

Amendments

Claims 17 and 23 have been amended to delete the term "prevention and/or" from the claims.

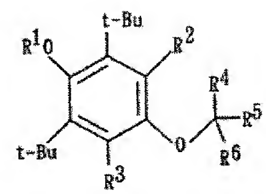
New claims 32-34 have been added. Support for claims 32 and 33 can be found in the specification as filed at paragraph 0013. Support for claim 34 can be found in the specification as filed at paragraph 0033.

Art Rejections

Claims 17-31 are rejected under 35 U.S.C. 103(a) s being unpatentable over Tamura et al., US 5,574,178, in view of Tsujii et al., US 5,043,354.

This rejection is respectfully traversed.

The compound disclosed in Tamura et al (US 5,574,178) is represented by formula (I):



(I)

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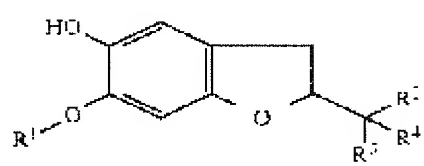
which covers the compound of claim 17 of the present application.

The benzofuran derivative disclosed in Tsujii et al (US 5,043,354) is represented by the formula:



(I)

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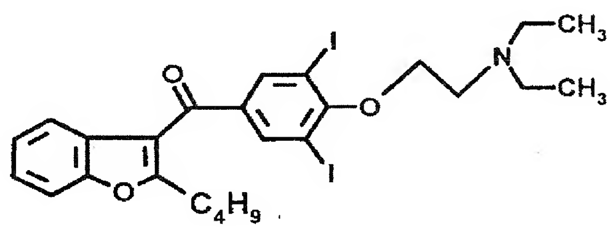
Those compounds are structurally common to each other in that they have a benzofuran ring when R^2 and R^4 in formula (I) of Tamura et al are taken together to form a 5-membered ring.

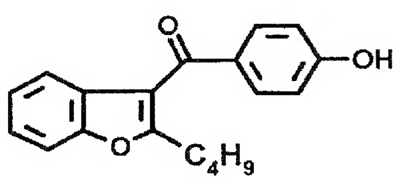
Regarding the pharmaceutical effect of benzofuran ring, Spaniol et al (Journal of Hepatology 35 (2001) 628-636, a copy of which is attached) shows that benzofuran ring of the amiodarone molecule, which is a highly effective antiarrhythmic agent, is responsible for liver mitochondrial toxicity caused by amiodarone, which toxicity is, in turn,

responsible for one of several adverse effects of amiodarone, hepatocellular liver damage.

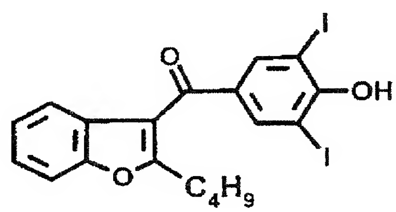
Briefly, Spaniol et al synthesized the four amiodarone analogues composed of different parts of the amiodarone molecule, B0, B2, C1 and D2, each having the following structures:



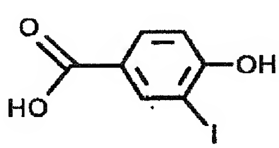
Amiodarone



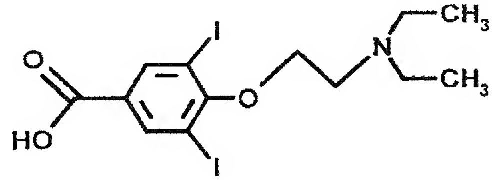
B 0



B 2



C 1



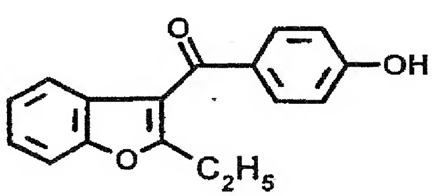
D 2

and investigated their effect on the function of isolated rat liver mitochondria, and concluded that the benzofuran structure is responsible for mitochondrial toxicity of

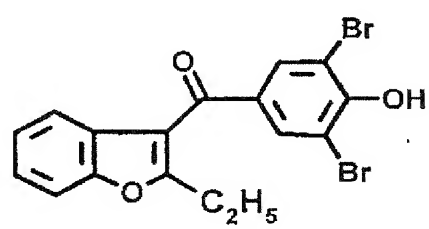
amiodarone and the presence of iodine is not essential. Refer to page 628, lines 13-15; page 628, right column, line 14 to page 629, left column, line 4; and page 629, right column, line 44 to the bottom of page 630.

The conclusion reached by Spaniol et al is supported by the other group, Quaglino et al (Am J Physiol Lung Cell Mol Phyiol 287: L438-L447, 2004, a copy of which is attached). Quaglino et al also indicates that the benzofuran moiety of amiodarone is toxic to liver cells. Refer to page L438, left column, lines 25-26.

Spaniol et al further shows the structures of benzarone and benzbromarone:

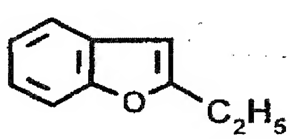


Benzarone

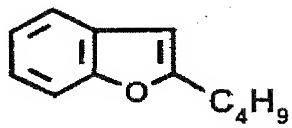


Benzbromarone

as drugs containing a structure similar to amiodarone in that they have 2-ethyl-benzofuran group:



which is closed to 2-butyl-benzofuran group contained in amiodarone:



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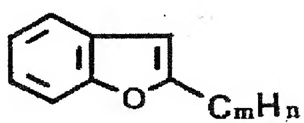
and as those causing serious hepatic injury. Refer to the bottom of Fig. 1 depicted on page 630 and the description appearing on page 634, right column, lines 3 to 16.

This indicates that the benzofuran structure or benzofuran ring structure referred to in Spaniol et al as being responsible for mitochondrial toxicity can be generalized to "2-alkyl-benzofuran group":



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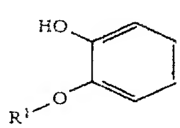
As the Examiner points out, Tsujii et al suggests that the benzofuran derivative disclosed therein is useful for the treatment of disorders of liver. Comparing the benzofuran derivative of Tsujii et al with the 2-alkyl-benzofuran group mentioned above, the benzofuran derivative is unique in that it has a "catechol unit":



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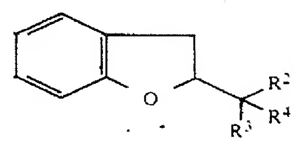
In view of the fact that the benzofuran derivative disclosed in Tsujii et al has 2-branched alkyl-benzofuran group:



which is closed to 2-alkyl-benzofuran group and is considered to also cause a mitochondrial or hepatic toxicity according to the conclusion reached by Spaniol et al, one skilled in the art would have ascribed the activity of the benzofuran derivative disclosed in Tsujii et al to treat a hepatic disease to the catechol unit.

Referring to the structure of the compound disclosed in Tamura et al, which corresponds to the compound of claim 17 of the present application when R^2 and R^4 are taken together to form a 5-membered ring, it does not contain catechol unit.

Therefore, one skilled in the art would not have expected from the disclosures of Tamura et al and Tsujii et al, that the compounds of claims 17-31 are useful for the treatment of fatty liver or hepatic disease, before the priority date of the present application and, thus, claims 17-31 are not obvious over Tamura et al in view of Tsujii et al.

In view of the above, it is respectfully submitted that the claims are now in condition for allowance, and favorable action thereon is earnestly solicited.

Respectfully submitted,

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Journal of Hepatology 35 (2001) 628–636

Journal of Hepatology

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Toxicity of amiodarone and amiodarone analogues on isolated rat liver mitochondria

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Background: **Amiodarone is a well-known mitochondrial toxin consisting of a benzofuran ring (ring A) coupled to a p-OH-benzene structure substituted with 2 iodines and a diethyl-ethanolamine side chain (ring B).**

Aim: **To find out which part of amiodarone is responsible for mitochondrial toxicity.**

Methods: **Amiodarone, ring A and B without the ethanolamine side-chain and iodines (B0), ring A and B with iodines but no ethanolamine (B2), ring B with 1 iodine and no ethanolamine (C1) and ring B with ethanolamine and 2 iodines (D2) were studied.**

Results: **In freshly isolated rat liver mitochondria, amiodarone inhibited state 3 glutamate and palmitoyl-CoA oxidation and decreased the respiratory control ratios. B0 and B2 were more potent inhibitors than amiodarone and B2 more potent than B0. C1 and D2 showed no significant mitochondrial toxicity. After disruption, mitochondrial oxidases and complexes of the electron transport chain were inhibited by amiodarone, B0 and B2, whereas C1 and D2 revealed no inhibition. Beta-oxidation showed a strong inhibition by amiodarone, B0 and B2 but not by C1 or D2. Ketogenesis was almost unaffected.**

Conclusions: **Amiodarone, B0 and B2 are uncouplers of oxidative phosphorylation, and inhibit complexes I, II and III, and β-oxidation. The benzofuran structure is responsible for mitochondrial toxicity of amiodarone and the presence of iodine is not essential.**

Keywords: **Amiodarone; Mitochondria; Electron transport chain; β-Oxidation; Ketogenesis; Liver injury**

1. Introduction

Amiodarone is a highly effective antiarrhythmic agent with class III activity according to the classification of Vaughan-Williams [1]. It is used in the treatment and prophylaxis of both ventricular and supraventricular arrhythmias [1], in particular in patients with heart insufficiency [2,3], because it has no significant negative inotropic effect.

Amiodarone has several adverse effects including corneal deposits, hypo- and hyperthyreosis, pulmonary fibrosis and hepatocellular liver damage [1]. About 1–3% of the patients treated with amiodarone suffer from symptomatic liver disease which may histologically be similar to alcoholic liver lesions [4,5]. One of the reasons for hepatic toxicity of amiodarone may be impairment of the function of liver mitochondria. It has been shown in mice that amiodarone inhibits mitochondrial β-oxidation of fatty acids, leading to microvesicular steatosis of the liver [6]. In addition, amiodarone has an uncoupling effect on oxidative phosphorylation and inhibits the function of the electron transport chain in isolated mice liver mitochondria [7].

The current study was undertaken to find out which part of the amiodarone molecule is responsible for mitochondrial toxicity. Amiodarone is composed of a diiodobenzene ring carrying a diethylaminoethoxy side chain, and a benzofuran ring carrying a C_4H_9 side chain (Fig. 1). In order be able to

Received 6 April 2001; received in revised form 17 July 2001; accepted 26 July 2001

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Abbreviations: CoASH, coenzyme A; complex I, NADH:ubiquinone-1 oxidoreductase; complex II, succinate:dichlorindophenol oxidoreductase; complex III, ubiquinol:ferricytochrome *c* oxidoreductase; complex IV, cytochrome oxidase; complex V, F_1F_0-ATPase; DMSO, dimethylsulfoxide; RCR, respiratory control ratio.

0168-8278/01/$20.00
PII: S0168-8278(01)00189-1

answer our question, we synthesized four amiodarone analogues composed of different parts of the amiodarone molecule and investigated their effect on the function of isolated rat liver mitochondria.

2. Materials and methods

2.1. Reagents

Amiodarone hydrochloride and the amiodarone analogues were synthesized as described previously [8]. Duroquinol (durohydroquinone) was purchased from ReseaChem GmbH (Burgdorf, Switzerland). [1-^{14}C]Palmitic acid was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland). All other chemicals were purchased from Sigma Chemical Co. (Buchs, Switzerland).

2.2. Animals

Male Sprague–Dawley rats were purchased from BRL Biological Research (Füllinsdorf, Switzerland). They were fed ad libitum and held on a 12:12-h dark/light cycle. The study protocol had been accepted by the Animal Ethics Committee of the Canton Basel Stadt.

2.3. Isolation of liver mitochondria

Rats were killed by decapitation and mitochondria were isolated from the liver by differential centrifugation according to the method of Hoppel et al. [9]. This method yields mitochondria of high purity with only minor contamination by peroxisomes or lysosomes [10]. The mitochondrial protein content was determined using the biuret method with bovine serum albumin as a standard [11].

2.4. Oxygen consumption by intact mitochondria

Oxygen consumption was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C as described previously [12].

The respiratory control ration (RCR) was determined as a marker of the functional integrity, e.g. coupling of oxidative phosphorylation, of the mitochondria. The RCR is the ratio of the rate of the oxygen consumption in the presence of a substrate and ADP (state 3) to the rate after complete conversion of ADP to ATP (state 4) [13].

The concentration of the substrates used were 20 mmol/l for L-glutamate and 80 μmol/l for palmitoyl-CoA. The incubations with palmitoyl-CoA contained also 2 mmol/l L-carnitine and 5 mmol/l L-malate.

Amiodarone and the analogues were dissolved in dimethylsulfoxide (DMSO) and added to the mitochondrial incubations before addition of the respective substrate at final concentrations of 10–100 μmol/l as indicated in the Tables 1–4. Control incubations contained the same amount of DMSO as the incubations with amiodarone or analogues.

2.5. Activities of mitochondrial oxidases

The activity of NADH, succinate, duroquinol and cytochrome c oxidase were determined at 30°C with the oxygen electrode using freeze-thawed mitochondria as described originally by Blair et al. [14] and with the modifications described previously [12].

2.6. Activities of the enzyme complexes of the electron transport chain and of long-chain acyl-CoA dehydrogenase

The activities of complex I–IV of the electron transport chain were determined by spectrophotometric methods as described previously for complexes I, II, IV [12], and III [15].

The activity of long-chain acyl-CoA dehydrogenase was determined using the spectrophotometric method of Hoppel et al. [9]. In this assay, palmitoyl-CoA is used as a substrate and the reduction of cytochrome c is monitored.

2.7. In vitro mitochondrial β-oxidation

The β-oxidation of [1-^{14}C] palmitic acid by liver mitochondria was assessed as described by Fréneaux et al. [5] with some modifications. The preincubation medium (900 μl of 70 mmol/l sucrose, 43 mmol/l KCl, 3.6 mmol/l $MgCl_2$, 7.2 mmol/l potassium phosphate and 36 mmol/l Tris–HCl, pH 7.4) contained 0.2 mmol/l ATP, 50 μmol/l L-carnitine, 15 μmol/l coenzyme A, 1 mg mitochondrial protein and 5 mmol/l acetoacetate.

After 5 min of preincubation at 30°C the incubation mixture was brought to 1 ml by adding 100 μl of preincubation buffer containing [1-^{14}C]palmitic acid (final concentration 40 μmol/l; 0.05 μCi per ml) and bovine serum albumin (final concentration 2.5 mg/ml). The incubations were carried out for 15 min at 30°C with slow shaking. Pilot studies had shown that the reaction was in the linear phase at this time point. The reaction was stopped by adding 100 μl of 30% perchloric acid (w/v) and by putting the tubes on ice for 20 min. The incubation vials were then centrifuged and the ^{14}C-acid-soluble β-oxidation products were counted in the supernatant. Such products represent mainly ketone bodies and citric acid cycle intermediates [5].

2.8. Determination of ketone body formation

Ketone body formation by liver mitochondria was measured in freeze-thawed mitochondria according to Chapman et al. [16] with some modifications.

Two mg of mitochondrial protein were incubated at 37°C for 15 min with an acetyl-CoA generating system in a final volume of 900 μl. This system contained 25 units of phosphotransacetylase and the following components at the final concentrations indicated: 10 mmol/l sodium phosphate (pH 7.4), 4 mmol/l ATP, 30 mmol/l lithium acetylphosphate, 1 mmol/l CoASH, 35 mmol/l KCl, 3 mmol/l $MgCl_2$ and 0.5 mmol/l dithiothreitol. The reaction was stopped by adding 100 μl of 30% perchloric acid (w/v). After having removed the precipitate by centrifugation, the supernatants were analyzed for acetoacetate according to Olsen [17].

2.9. Statistical methods

Data are presented as mean ± SD. Differences between control incubations and incubations containing amiodarone or amiodarone analogues were analyzed using analysis of variance (ANOVA) for repeated measures and Tukey's protected *t*-test to localize differences obtained by the ANOVA. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

The toxicity of amiodarone and the structural analogues B0, B2, C1 and D2 (structures shown in Fig. 1) was tested in isolated rat liver mitochondria. The effect of amiodarone and analogues on oxidative mitochondrial metabolism using glutamate or palmitoyl-CoA as substrate is shown in Table 1. In the presence of glutamate as substrate, amiodarone, B0 and B2 revealed a dose dependent toxic effect on mitochondrial metabolism. Glutamate is metabolized by glutamate dehydrogenase to α-ketoglutarate and NADH which is oxidized by complex I of the electron transport chain (Fig. 2). Complex I transfers the electrons to

complexes III and IV. These substances inhibited state 3 oxidation, reflecting impairment of the function of the electron transport chain. The corresponding IC_{50} values were 83 ± 29 μmol/l for amiodarone, 57 ± 40 μmol/l for B0 and 17 ± 6 μmol/l for B2. Furthermore, they increased state 4 oxidation (results not shown, for calculation see Table 1), leading to a decrease of the respiratory control ratio (RCR) and suggesting uncoupling of the activity of the electron transport chain from production of ATP. The IC_{50} values for the decrease in RCR were 13 ± 5 μmol/l for

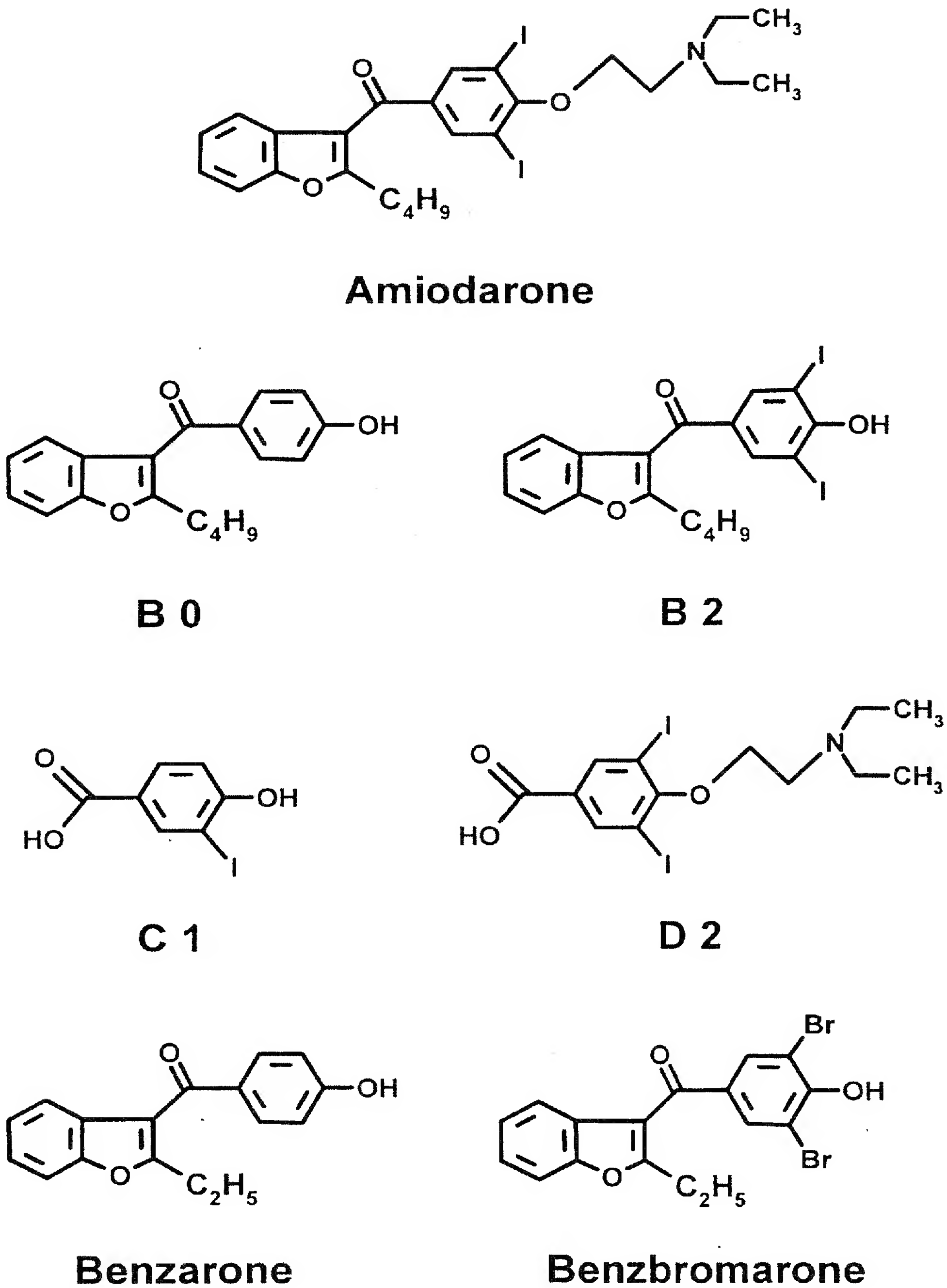

Fig. 1. Chemical structure of amiodarone analogues. B0 and B2 are the result of *O*-dealkylation of amiodarone. B0 carries no iodine atoms at the benzofuran group. D2 and C1 are the result of removal of the 2-butyl-benzofuran group of the amiodarone molecule. C1 has also undergone *O*-dealkylation. Benzbromarone and benzarone are two drugs with a chemical structure similar to amiodarone.

Table 1
Oxidative metabolism of glutamate and palmitoyl-CoA by isolated rat liver mitochondria[a]

	L-Glutamate (20 mmol/l)		Palmitoyl-CoA	
	State 3	RCR	State 3	RCR
Control (no inhibitor)	92 ± 5	6.6 ± 0.7	35 ± 5	2.5 ± 0.3
Amiodarone (μmol/l)				
10	82 ± 6	3.0 ± 0.2**	59 ± 22*	2.2 ± 0.2
20	71 ± 4	2.6 ± 0.2**	61 ± 11*	2.0 ± 0.1*
50	56 ± 29*	1.0 ± 0**	47 ± 17	1.9 ± 0.4
100	0**	–	4.8 ± 2.3**	1.0 ± 0**
B0 (μmol/l)				
10	71 ± 5*	3.2 ± 0.4**	45 ± 6	2.2 ± 0.4
20	55 ± 13**	1.7 ± 0.4**	54 ± 3*	1.9 ± 0.3*
50	32 ± 12**	1.0 ± 0**	18 ± 17	1.5 ± 0.3*
100	0**	–	3.7 ± 2.6**	1.0 ± 0**
B2 (μmol/l)				
10	55 ± 11**	1.0 ± 0**	55 ± 6	2.9 ± 0.4
20	42 ± 3**	1.0 ± 0**	51 ± 30	2.0 ± 0.8
50	19 ± 6**	1.0 ± 0**	14 ± 9	1.0 ± 0**
100	0**	–	0**	–
C1 (μmol/l)				
10	65 ± 12**	5.0 ± 1.5	48 ± 11	2.1 ± 0.2
20	84 ± 9	7.0 ± 2.3	42 ± 4	2.4 ± 0.7
50	72 ± 5*	5.5 ± 0.4	37 ± 6	2.2 ± 0.5
100	76 ± 5*	5.8 ± 0.7	36 ± 7	2.1 ± 0.5
D2 (μmol/l)				
10	79 ± 10	5.6 ± 0.4	30 ± 1	2.0 ± 0.2
20	74 ± 12*	7.2 ± 1.4	31 ± 5	2.4 ± 0.2
50	78 ± 2	8.8 ± 0.4	34 ± 10	2.6 ± 0.4
100	84 ± 7	6.9 ± 1.5	32 ± 5	2.3 ± 0.2

[a] Units for state 3 activity are natoms/min/mg mitochondrial protein. The respiratory control ratio (RCR) is calculated by dividing state 3 by state 4 respiration (state 4 respiration is not given). The structure of the amiodarone analogues is given in Fig. 1. Data are given as mean ± SD of three individual mitochondrial preparations. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control.

amiodarone, 17 ± 6 μmol/l for B0 and <10 μmol/l for B2. Considering the IC_{50} values, B2 appeared to be slightly more toxic than amiodarone or B0, suggesting that the two iodines are not essential for mitochondrial toxicity but enhance the toxicity of amiodarone and analogues. In contrast to B0 and B2, C1 and D2 revealed only a slight decrease of the activity of the electron transport chain without clear dose dependency.

The stimulation of state 4 respiration could be due to uncoupling of oxidative phosphorylation (direct transport of protons across the inner mitochondrial membrane by bypassing F_1F_0-ATPase) or by a decrease of the intramitochondrial ATP level, stimulating secondarily the entry of protons through F_1F_0-ATPase [18]. To differentiate between these two possibilities, oligomycin, an inhibitor of F_1F_0-ATPase, was added during state 4 in the presence of glutamate as substrate and amiodarone or analogues. Inhibition of F_1F_0-ATPase should prevent the observed increase in state 4 oxidation, if this were a result of mitochondrial consumption of ATP. However, oligomycin (final concentration 5 μg/ml) did not prevent the stimulation of state 4 oxygen consumption (basal value 14 ± 3 natoms/min per mg) by 10 μmol/l amiodarone (state 4 oxidation rates 28 ± 4 vs. 30 ± 6 natoms/min per mg in the presence or absence of oligomycin, respectively), 10 μmol/l B0 (23 ± 5 vs. 22 ± 3 natoms/min per mg) or by 10 μmol/l B2 (53 ± 9 vs. 56 ± 8 natoms/min per mg). These findings indicate that the increase in state 4 respiration induced by amiodarone, B0 or B2 is due to uncoupling of oxidative phosphorylation, e.g. due to a protonophoric effect of these substances.

Palmitoyl-CoA is metabolized by β-oxidation which yields NADH, FADH and acetyl-CoA. NADH is oxidized by complex I and FADH by the electron transferring flavoprotein (ETF)/ETF dehydrogenase. From complex I and ETF dehydrogenase the electrons are transferred to ubiquinone, and from ubiquinone to complex III and IV (see Fig. 2). Amiodarone stimulated metabolism of palmitoyl-CoA at low concentrations but inhibited state 3 oxidation rates and decreased the RCR at higher concentrations. B0 and B2

Table 2
Activities of mitochondrial oxidases[a]

	NADH	Succinate	Duroquinol	Cytochrome *c*
Control (no inhibitor)	364 ± 38	98 ± 33	546 ± 121	460 ± 44
Amiodarone (μmol/l)				
10	303 ± 78	79 ± 41	479 ± 96	409 ± 47
20	294 ± 27*	90 ± 42	510 ± 110	391 ± 62
50	305 ± 61	88 ± 43	504 ± 157	418 ± 64
100	269 ± 27*	84 ± 41	464 ± 157	366 ± 38*
B0 (μmol/l)				
10	221 ± 12**	99 ± 37	495 ± 148	418 ± 33
20	153 ±18**	95 ± 44	475 ± 182	458 ± 23
50	108 ± 10**	73 ± 41*	370 ± 168*	468 ± 23
100	65 ± 6**	47 ± 19**	300 ± 120**	529 ±31
B2 (μmol/l)				
10	268 ± 48**	63 ± 24**	510 ± 120	441 ± 30
20	176 ± 16**	38 ± 13**	469 ±108	487 ± 25
50	123 ± 42**	19 ± 9**	300 ± 40**	591 ± 131
100	66 ± 12**	4 ± 4**	157 ± 40**	791 ± 112**
C1 (μmol/l)				
10	337 ± 25	88 ± 3	537 ± 155	430 ± 45
20	333 ± 20	88 ± 14	543 ± 174	429 ± 45
50	337 ± 69	98 ± 35	534 ± 193	435 ± 66
100	333 ± 50	96 ± 27	498 ± 141	409 ± 66
D2 (μmol/l)				
10	304 ± 16*	100 ± 39.3	503 ± 106.8	426 ± 56
20	302 ± 33	99 ± 34.2	500 ± 69.3	415 ± 81
50	305 ± 12*	101 ± 39.9	518 ± 82.2	445 ± 81
100	297 ± 3*	89 ± 18.9	513 ± 75.7	417 ± 34

[a] Activities are expressed as natoms/min/mg mitochondrial protein. The structure of the amiodarone analogues is given in Fig. 1. Data are given as mean ± SD of three individual mitochondrial preparations. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

showed a similar behavior, leading to an almost complete inhibition of state 3 oxidation at 100 μmol/l. In contrast, C1 and D2 had no toxic effects on mitochondrial metabolism up to 100 μmol/l.

In order to exclude the possibility that the observed inhibition of the state 3 oxidation rates was caused by inhibition of dehydrogenation of glutamate and/or β-oxidation of palmitoyl-CoA, and to localize the inhibitory effects of amiodarone and analogues on the electron transport chain in more detail, the activity of the mitochondrial oxidases were studied. Mitochondrial oxidases are measured with broken mitochondria and the substrates used can therefore interact directly with the complexes of the electron transport chain. As shown in Table 2, amiodarone, and in particular B0 and B2 showed a strong concentration dependent inhibition of NADH oxidase (which involves complex I, III and IV) and succinate oxidase (which involves complex II, III and IV). Duroquinol oxidase (involving complex III and IV) was significantly inhibited only by B2 and B0 (but not amiodarone), but to a lower degree than NADH or succinate oxidase. In contrast, cytochrome *c* oxidase was not inhibited by amiodarone or by any of the analogues. C1 did not inhibit any of the mitochondrial oxidases (suggesting that the observed reduction of L-glutamate metabolism by C1 was due to inhibition of glutamate dehydrogenase). D2 showed a slight inhibition of NADH but of none of the other oxidases, indicating that D2 is a weak inhibitor of complex I.

In order to determine the effect of amiodarone, B0 and B2 on complex I, II and III more precisely, these enzyme complexes were investigated individually using broken mitochondria. As shown in Table 3, amiodarone and both analogues inhibited complex I and II in a concentration-dependent way. Similar to the data in Tables 1 and 2, B2 appeared to be a more potent inhibitor than amiodarone or B0. Regarding complex III, a significant inhibition was only found for B2 but not for amiodarone or B0.

The results so far obtained show that amiodarone, B0 and B2 inhibit enzyme complexes of the electron transport chain and uncouple oxidative phosphorylation. Since amiodarone has been shown to inhibit mitochondrial β-oxidation [6], we investigated the effect of amiodarone and analogues on mitochondrial β-oxidation and ketogenesis (Table 4). Amiodarone, B0 and B2 impaired the mitochondrial formation of acid-soluble products, which reflects both, β-oxida-

Table 3
Activities of enzyme complexes of the electron transport chain in isolated rat liver mitochondria[a]

	Complex I	Complex II	Complex III
Control (no inhibitor)	20.1 ± 0.6	8.2 ± 2.5	257 ± 171
Amiodarone (μmol/l)			
10	13.6 ± 6.7	5.8 ± 0.6	257 ± 48
20	12.4 ± 4.7	4.1 ± 2.6*	256 ± 40
50	8.9 ± 7.3*	2.9 ± 1.8**	348 ± 57
80	7.7 ± 6.4*	2.3 ± 1.3**	387 ± 142
100	6.9 ± 5.4*	2.3 ± 1.9**	234 ± 219
B0 (μmol/l)			
10	12.0 ± 6.7*	7.1 ± 0.5	232 ± 143
20	11.0 ± 9.7 *	5.9 ± 1.5	278 ± 52
50	13.9 ± 5.5	2.9 ± 0.8**	253 ± 98
80	7.4 ± 1.3**	3.9 ± 2.3**	205 ± 107
100	6.8 ± 0.7**	0.5 ± 0.3**	241 ± 75
B2 (μmol/l)			
10	18.0 ± 6.9	13.9 ± 1.7*	209 ± 98
20	7.4 ± 1.1**	6.3 ± 4.7	225 ± 75
50	5.0 ± 6.4**	0.8 ± 1.1*	149 ± 32*
80	5.6 ± 4.5**	0.0 ± 0**	160 ± 38*
100	3.1 ± 2.5**	0.0 ± 0**	139 ± 16*

[a] Activities of the enzyme complexes are expressed as mU/mg mitochondrial protein. The structure of the amiodarone analogues is given in Fig. 1. Data are given as mean ± SD of three individual mitochondrial preparations. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control.

tion and ketogenesis. Since the activity of the respiratory chain can be rate-limiting for mitochondrial fatty acid metabolism [19], acetoacetate was added to the incubations. Acetoacetate converts NADH to NAD by the action of β-hydroxybutyrate dehydrogenase (an enzyme of the mitochondrial matrix) and thereby eliminates the indirect impairment of mitochondrial β-oxidation by inhibitors of the electron transport chain (such as amiodarone and analogues) [6]. Ketogenesis was inhibited weakly only by B0, but not by amiodarone or B2, indicating that the observed inhibition of the production of acid soluble products can be explained almost entirely by reduced β-oxidation. This was further investigated by measuring the activity of long-chain acyl-CoA dehydrogenase, the first enzyme of the mitochondrial β-oxidation cycle and an enzyme which can be rate limiting for this cycle [20]. This enzyme was inhibited by amiodarone, B0 and B2, showing directly that mitochondrial β-oxidation is impaired by amiodarone and analogues. In comparison, C1 had no inhibitory effects on formation of acid soluble products and ketogenesis, whereas D2 inhibited the formation of acid soluble products only weakly but had no effect on ketogenesis.

4. Discussion

Our study shows that amiodarone, B0 and B2 are uncouplers of oxidative phosphorylation, inhibit various enzyme complexes of the electron transport chain and also mitochondrial β-oxidation. The essential structure associated with mitochondrial toxicity appears to be the benzofuran ring, whereas the presence of iodine is not essential for mitochondrial toxicity of amiodarone and analogues.

As shown in Table 1, amiodarone, B0 and B2 are more potent as uncouplers than as inhibitors of enzyme complexes of the respiratory chain. The protonophoric effect of amiodarone has been explained by protonation of the tertiary amine in the intermembranaceous space (space between outer and inner membrane of mitochondria), transport of the resulting quarternary amine across the inner mitochondrial membrane, deprotonation in the mitochondrial matrix and diffusion of amiodarone back to the intermembranaceous space [18]. The uncoupling mechanism must obviously be different for B0 and B2, since neither of these molecules contains a nitrogen which can be protonated. These molecules are lipophilic and can therefore diffuse across the inner mitochondrial membrane. They are weak acids (hydroxyl group at the benzene ring), and can therefore be deprotonated in the basic milieu of the mitochondrial matrix. By both mechanisms, protons are transported into the mitochondrial matrix by bypassing the F_1F_0-ATPase. Interestingly, D2 and C1 had no uncoupling effect despite they also contain a tertiary amine (D2), a carboxyl group (D2 and C1) or a hydroxyl group attached to a benzene ring (C1). Not only the functional groups but also other properties such as e.g. lipophilicity, K_a and/or K_b values, which were not investigated in the current studies, may therefore be important for the uncoupling capacity of a molecule.

Beside their uncoupling effect, amiodarone inhibits complex I and II, and B0 and B2 complex I, II and also weakly complex III. In contrast, complex IV was not significantly inhibited by any of the substances. Since ubiquinone is the electron acceptor of both, complex I and II and transfers the electrons to complex III (see Fig. 2), it is possible that amiodarone and analogues interfere somehow with the electron transport between complexes I or II and complex III. The observed reduction in the activities of the individual enzyme complexes (see Table 3) does not exclude this possibility, since ubiquinones act as electron acceptors (assay for complex I) or as electron transmitters (assay for complex II) in the enzyme assays used.

Besides their effect on the respiratory chain, amiodarone, B0 and B2 inhibit mitochondrial β-oxidation independently. Since the activity of the electron transport chain can be rate-limiting for mitochondrial fatty acid metabolism [19], it was important to eliminate the possibility that the observed inhibition of β-oxidation is secondary to impaired function of the electron transport chain by amiodarone, B2 or B0, by including acetoacetate into the incubations. Since ketogenesis was not (amiodarone, B0) or only weakly inhibited (B2), we could show convincingly that amiodarone and analogues inhibit the β-oxidation cycle directly. This notion

Table 4
Influence of amiodarone and analogues on mitochondrial β-oxidation and ketogenesis[a]

	β-Oxidation	Palmitoyl-CoA dehydrogenase	Ketogenesis
Control (no inhibitor)	0.66 ± 0.23	36.2 ± 3.1	14.0 ± 0.9
Amiodarone (μmol/l)			
10	0.50 ± 0.06	32.6 ± 1.2	12.1 ± 10.3
20	0.44 ± 0.10*	24.8 ± 3.2**	13.5 ± 7.1
50	0.03 ± 0.03**	13.1 ± 1.9**	10.0 ± 19.3
80	0.03 ± 0.02**	15.3 ± 1.0**	14.4 ± 12.4
100	0.03 ± 0.02**	21.8 ± 1.4**	9.9 ± 6.2
B0 (μmol/l)			
10	0.95 ± 0.11**	36.2 ± 9.2	10.9 ± 1.6
20	0.72 ± 0.05	28.8 ± 3.1*	13.6 ± 3.0
50	0.03 ± 0.01**	27.0 ± 6.7*	6.9 ± 3.0*
80	0.02 ± 0.01**	21.7 ± 2.6**	11.2 ± 2.1
100	0.02 ± 0.01**	11.6 ± 1.1**	8.0 ± 3.2*
B2 (μmol/l)			
10	0.67 ± 0.11	34.7 ± 7.1	17.1 ± 4.6
20	0.70 ± 0.09	26.1 ± 3.2*	14.7 ± 4.8
50	0.63 ± 0.43	15.4 ± 7.2**	14.3 ± 4.8
80	0.03 ± 0.02**	12.4 ± 4.4**	12.8 ± 2.4
100	0.02 ± 0.02**	12.2 ± 4.0**	11.2 ± 2.2
C1 (μmol/l)			
10	0.73 ± 0.37		20.5 ± 3.1*
20	0.68 ± 0.36		19.1 ± 5.4
50	0.71 ± 0.37		17.4 ± 4.6
80	0.70 ± 0.39		13.4 ± 3.4
100	0.68 ± 0.33		18.5 ± 3.0
D2 (μmol/l)			
10	0.63 ± 0.20		12.2 ± 6.5
20	0.60 ± 0.25		11.9 ± 8.6
50	0.62 ± 0.23		10.9 ± 5.4
80	0.56 ± 0.26		11.4 ± 8.7
100	0.51 ± 0.01*		14.7 ± 5.7

[a] Metabolic and enzyme activities are given as nmol/min/mg mitchondrial protein. The structure of the amiodarone analogues is given in Fig. 1. Data are given as mean ± SD of three individual mitochondrial preparations. *$P < 0.05$ vs. no inhibitor; **$P < 0.01$ vs. no inhibitor.

is further supported by the inhibitory effect of amiodarone, B2 and B0 on the activity of long-chain acyl-CoA dehydrogenase. This effect can explain microvesicular hepatic steatosis associated with amiodarone, which is a well established consequence of drugs or toxins impairing mitochondrial β-oxidation [21,22].

Concerning the chemical structures of the molecules investigated, it is clear that iodination is not essential for mitochondrial toxicity of amiodarone and B2, since B0 contains no iodine. Regarding uncoupling of oxidative phosphorylation and inhibition of the respiratory chain, B2 appears to be a more potent toxin than B0, however, suggesting that iodination of the benzene ring in the amiodarone molecule may enhance toxicity on the mitochondrial respiratory chain. Since C1 and D2 caused no significant impairment of mitochondrial functions, the diethylaminoethoxy group and the benzene ring are not essential for mitochondrial toxicity of amiodarone and analogues. The common structure of amiodarone, B0 and B2 is the benzofuran ring structure, which therefore can be considered to be responsible for mitochondrial toxicity of amiodarone. This notion is supported by the fact that benzarone and benzbromarone, two drugs containing a benzofuran ring structure (see Fig. 1), can cause serious hepatic injury. Benzarone, used for the treatment of peripheral venous disorders, can cause chronic active hepatitis [23], in some patients with a fatal outcome [24]. Benzbromarone, an uricosuric agent whose structure differs from benzarone only by two additional brom atoms at the benzofuran ring, is also known to cause hepatic injury with possible fatal outcome [25]. In support of our hypothesis that the benzofuran ring mediates mitochondrial toxicity of these molecules, benzbromarone has been shown to inhibit several enzyme complexes of the electron transport chain in isolated rat liver mitochondria [26].

In conclusion, the benzofuran group appears to be the responsible for mitochondrial toxicity of amiodarone and

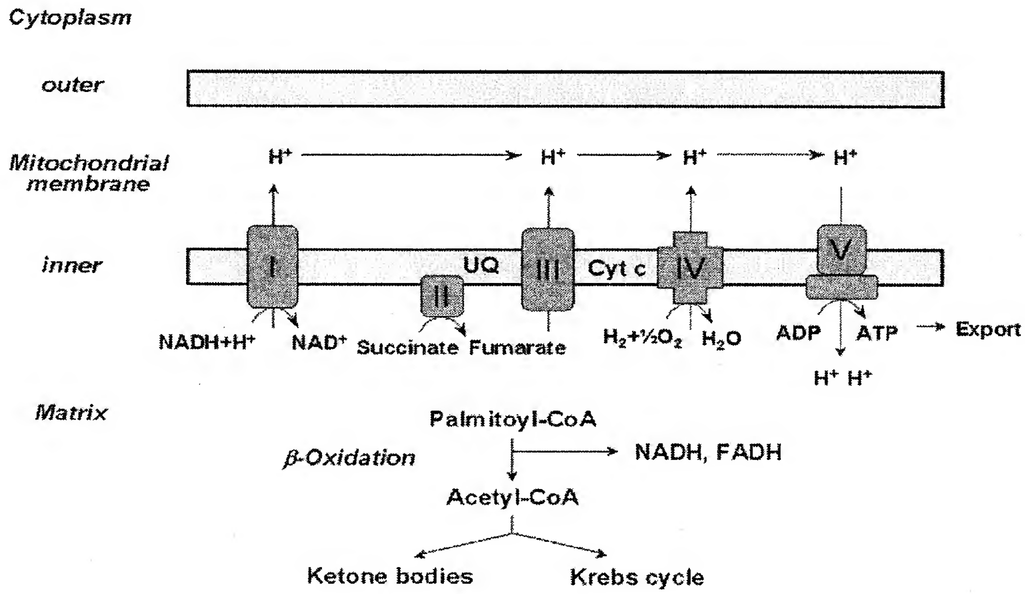

Fig. 2. Mitochondrial β-oxidation and function of the respiratory chain. The respiratory chain consists of 4 enzyme complexes (complexes I–IV) which transport electrons from NADH, succinate or FADH to oxygen, leading to the production of water. Ubiquinone and cytochrome *c* are electron shuttles which transport electrons between complexes I or II and III, and complexes III and IV, respectively. Complexes I, III and IV transport protons from the mitochondrial matrix into the intermembranaceous space, leading to a proton gradient across the inner mitochondrial membrane. Protons flow back into the mitochondrial matrix by F_1F_0-ATPase, leading to the production of ATP from ADP. Uncouplers of the respiratory chain enable protons to flow back to the mitochondrial matrix without passing across F_1F_0-ATPase. Long-chain fatty acids are metabolized within mitochondria by β-oxidation which includes the action of the 4 enzymes acyl-CoA dehydrogenase, enoyl-CoA hydratase, β-hydroxy-acyl-CoA dehydrogenase and β-ketothiolase, and results in the production of acetyl-CoA, FADH and NADH. FADH and NADH are metabolized by the respiratory chain and most acetyl-CoA is converted to ketone bodies in liver mitochondria.

analogues. Taking into account reports from other drugs containing a bezofuran group, there are reasons to assume that this part of the amiodarone molecule represents the essential structure causing mitochondrial damage and possibly also liver injury in humans.

Acknowledgements

The study has been supported by grant 31-59812.99 from the Swiss National Science Foundation to S.K.

References

[1] Mason JW. Amiodarone. N Engl J Med 1987;316:455–466.
[2] Amiodarone Trials Meta-Analysis Investigators. Effect of prophylactic amiodarone on mortality after acute myocardial infarction and in congestive heart failure: meta-analysis of individual data from 6500 patients in randomised trials. Lancet 1997;350:1417–1424.
[3] Julian DG, Camm AJ, Frangin G, Janse MJ, Munoz A, Schwartz PJ, Simon P. Randomised trial of effect of amiodarone on mortality in patients with left-ventricular dysfunction after recent myocardial infarction: EMIAT. European Myocardial Infarct Amiodarone Trial Investigators. Lancet 1997;349:667–674.
[4] Lewis JH, Ranard RC, Caruso A, Jackson LK, Mullick F, Ishak KG, et al. Amiodarone hepatotoxicity: prevalence and clinicopathologic correlations among 104 patients. Hepatology 1989;9:679–685.
[5] Morse RM, Valenzuela GA, Greenwald TP, Eulie PJ, Wesley RC, McCallum RW. Amiodarone-induced liver toxicity. Ann Intern Med 1988;109:838–840.
[6] Fromenty B, Fisch C, Labbe G, Degott C, Deschamps D, Berson A, et al. Amiodarone inhibits the mitochondrial β-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. J Pharmacol Exp Ther 1990;255:1371–1376.
[7] Fromenty B, Fisch C, Berson A, Letteron P, Larrey D, Pessayre D. Dual effect of amiodarone on mitochondrial respiration. Initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and II. J Pharmacol Exp Ther 1990;255:1377–1384.
[8] Ha HR, Stieger B, Grassi G, Altorfer HR, Follath F. Structure–effect relationships of amiodarone analogues on the inhibition of thyroxine deiodination. Eur J Clin Pharmacol 2000;55:807–814.
[9] Hoppel C, DiMarco JP, Tandler B. Riboflavin and rat hepatic cell structure and function. Mitochondrial oxidative metabolism in deficiency states. J Biol Chem 1979;254:4164–4170.
[10] Gornall AG, Bardawill GJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem 1949;177:751–766.
[11] Krähenbühl S, Talos C, Reichen J. Mechanisms of impaired hepatic fatty acid metabolism in rats with long-term bile duct ligation. Hepatology 1994;19:1272–1281.
[12] Krähenbühl S, Chang M, Brass EP, Hoppel CL. Decreased activities of ubiquinol: ferricytochrome c oxidoreductase (complex III and ferrocytochrome c: oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria. J Biol Chem 1991;266:20998–21003.
[13] Estabrook RW. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol 1967;10:41–47.

[14] Blair PV, Oda T, Green DE, Fernandez-Moran H. Studies on the electron transfer system. LIV. Isolation of the unit of electron transfer. Biochemistry 1963;2:756–763.

[15] Krähenbühl S, Talos C, Wiesmann U, Hoppel CL. Development and evaluation of a spectrophotometric assay for complex III in isolated mitochondria, tissues and fibroblasts from rats and humans. Clin Chim Acta 1994;230:177–187.

[16] Chapman MJ, Miller LR, Ontko JA. Localization of the enzymes of ketogenesis in rat liver mitochondria. J Cell Biol 1973;58:284–306.

[17] Olsen C. An enzymatic fluorometric micromethod for the determination of acetoacetate, β-hydroxybutyrate, pyruvate and lactate. Clin Chim Acta 1971;33:293–300.

[18] Berson A, Fau D, Fornacciari R, Degove-Goddard P, Sutton A, Descatoire V, et al. Mechanisms for experimental buprenorphine hepatotoxicity: major role of mitochondrial dysfunction versus metabolic activation. J Hepatol 2001;34:261–269.

[19] Latipaa PM, Karki TT, Hiltunen JK, Hassinen IE. Regulation of palmitoylcarnitine oxidation in isolated rat liver mitochondria. Role of the redox state of NAD(H). Biochim Biophys Acta 1986;875:293–300.

[20] Osmundsen H, Bremer J. A spectrophotometric procedure for rapid and sensitive measurements of beta-oxidation. Demonstration of factors that can be rate-limiting for beta-oxidation. Biochem J 1977;164:621–633.

[21] Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 1995;67:101–154.

[22] Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bar W, Krahenbuhl S. Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. N Engl J Med 1997;336:1142–1148.

[23] Babany G, Larrey D, Pessayre D, Degott C, Rueff B, Benhamou JP. Chronic active hepatitis caused by benzarone. J Hepatol 1987;5:332–335.

[24] Hautekeete ML, Henrion J, Naegels S, DeNeve A, Adler M, Deprez C, et al. Severe hepatotoxicity related to benzarone: a report of three cases with two fatalities. Liver 1995;15:25–29.

[25] van der Klauw MM, Houtman PM, Stricker BH, Spoelstra P. Hepatic injury caused by benzbromarone. J Hepatol 1994;20:376–379.

[26] Kramar R, Müller MM. Inhibition of enzymes on the internal mitochondrial membrane by benzbromarone. Experientia 1973;29:391–392.

Am J Physiol Lung Cell Mol Physiol 287: L438–L447, 2004.
First published April 9, 2004; 10.1152/ajplung.00434.2003.

Effects of metabolites and analogs of amiodarone on alveolar macrophages: structure-activity relationship

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Submitted 5 December 2003; accepted in final form 25 March 2004

Quaglino, Daniela, Huy Riem Ha, Elena Duner, Daniela Bruttomesso, Laurent Bigler, Ferenc Follath, Giuseppe Realdi, Andrea Pettenazzo, and Aldo Baritussio. Effects of metabolites and analogs of amiodarone on alveolar macrophages: structure-activity relationship. *Am J Physiol Lung Cell Mol Physiol* 287: L438–L447, 2004. First published April 9, 2004; 10.1152/ajplung.00434.2003.—Amiodarone, an antiarrhythmic drug toxic toward the lung, is metabolized through sequential modifications of the diethylaminoethoxy group to mono-*N*-desethylamiodarone (MDEA), di-*N*-desethylamiodarone (DDEA), and amiodarone-EtOH (B2-O-EtOH), whose effects on lung cells are unclear. To clarify this, we exposed rabbit alveolar macrophages to analogs with different modifications of the diethylaminoethoxy group and then searched for biochemical signs of cell damage, formation of vacuoles and inclusion bodies, and interference with the degradation of surfactant protein A, used as a tracer of the endocytic pathway. The substances studied included MDEA, DDEA, and B2-O-EtOH, analogs with different modifications of the diethylaminoethoxy group, fragments of the amiodarone molecule, and the antiarrhythmic agents dronedarone (SR-33589) and KB-130015. We found the following: *1*) MDEA, DDEA, and B2-O-EtOH rank in order of decreasing toxicity toward alveolar macrophages, indicating that dealkylation and deamination of the diethylaminoethoxy group represent important mechanisms of detoxification; *2*) dronedarone has greater, and KB-130015 has smaller, toxicity than amiodarone toward alveolar macrophages; and *3*) the benzofuran moiety, which is toxic to liver cells, is not directly toxic toward alveolar macrophages.

dronedarone; KB-130015

AMIODARONE (Fig. 1) is a class III antiarrhythmic agent used extensively to treat ventricular and supraventricular arrhythmias and to prevent atrial fibrillation in patients undergoing cardiac surgery (16).

Despite recognized efficacy and wide use, amiodarone poses problems to clinicians because of its peculiar pharmacokinetics and toxicity. After absorption, amiodarone leaves the serum compartment with a half-life of 10–20 h and accumulates in peripheral tissues to very high levels, so that when the dosing rate is reduced or halted, the accumulated amiodarone moves back into serum, resulting in a long terminal elimination half-life (18). The tissues where amiodarone accumulates most include adipose tissue, liver, skeletal muscle, lung, pancreas, thyroid gland, kidney, heart, skin, adrenal glands, testis, and lymph nodes. In the liver and other tissues, amiodarone undergoes dealkylation to mono-*N*-desethylamiodarone (MDEA), a derivative with antiarrhythmic activity, propensity to accumulate into tissues, and a half-life similar to those of the parent drug but with greater toxicity (18). MDEA can be further transformed by dealkylation to di-*N*-desethylamiodarone (DDEA), by deamination to amiodarone-EtOH (B2-O-EtOH), and by hydroxylation to n-3′-hydroxybutyl-*N*-desethylamiodarone, whose biological effects are presently unknown (unpublished observations). Further degradation of these compounds probably occurs, since several studies have reported an increased excretion of free iodine during amiodarone therapy (18). Several *P*450 cytochromes, such as 3A4, 2D6, 1A1, and 1A2, are involved in amiodarone degradation (17).

Amiodarone metabolites MDEA, DDEA, and B2-O-EtOH are formed in vivo through sequential modifications of the diethylaminoethoxy group, but the relevance of these changes to lung cells is unclear. In this paper, to clarify this point, we exposed rabbit alveolar macrophages to analogs of amiodarone with different modifications of the diethylaminoethoxy group (Fig. 2) and then searched for biochemical signs of cell damage, changes in morphology, and interference with uptake and degradation of surfactant protein A (SP-A), used as a tracer of the endocytic pathway (4). The substances studied included known metabolites of amiodarone, such as MDEA, DDEA, and B2-O-EtOH, analogs with different modifications of the diethylaminoethoxy group, fragments of the amiodarone molecule, and derivatives with proven antiarrhythmic activity, such as dronedarone (SR-33589) and KB-130015 (7, 21). The effect of dronedarone and KB-130015 on the degradation of SP-A was studied in vivo as well, by administering them through the trachea to 3-day-old rabbits together with labeled SP-A and then following the disappearance of label from the airways and from the lungs.

MATERIALS AND METHODS

These experiments were approved by the local committee on the handling of laboratory animals.

Materials. All reagents were of analytical grade. Sodium ^{125}I was from Amersham Pharmacia Biotech (Little Chalfont, UK).

Drugs. Amiodarone·hydrochloride was from Sigma. MDEA·hydrochloride [2-*n*-Butyl-3-(3,5-diiodo-4-ethylaminoethoxybenzoyl)-benzofuran] was a gift from Sanofi (Munchenstein, Switzerland). KB-130015

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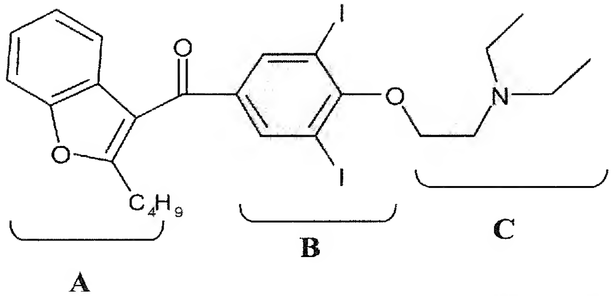


Fig. 1. Structure of amiodarone: benzofuran moiety with butyl lateral group (*A*), diiodobenzoyl moiety (*B*), and diethylamino-β-ethoxy moiety with tertiary nitrogen (*C*).

was a gift from Dr. Bo Carlsson (Caro Bio, Novum, Huddinge, Sweden). Dronedarone (SR-33589) was a gift from Sanofi-Synthelabo (Chilly-Mazarin Cedex, France). MeAMI {(2-butyl-benzofuran-3-yl)-4-[2-(di-methylamino-ethoxy)-3,5-diiodophenyl]-methanone}, DDEA {(2-butyl-benzofuran-3-yl)-4[2-(aminoethoxy)-3,5-diiodophenyl]-methanone·hydrochloride}, B2-O-AcOH [2-*n*-butyl-3-(3,5-diiodo-4-carboxyme-thoxybenzoyl)benzofuran], B2-O-EtOH, B2-O-Et {(2-butyl-benzofuran-3-yl)-(4-ethoxy-3,5-diiodophenyl)-methanone}, B2 [(2-butyl-benzo-furan-3-yl)-(4-hydroxy-3,5-diiodophenyl)-methanone], D2 [4-(2-diethylaminoethoxy)-3,5-diiodobenzoic acid], and DIB-O-A (4-carboxymethoxy-3,5-diiodobenzoic acid) were synthesized in the Cardiovascular Therapy Research Laboratory of the Zurich University Hospital (13). The chemical structure of newly synthesized compounds was confirmed by UV, liquid chromatography-electrospray ionization mass spectroscopy/mass spectroscopy, and nuclear magnetic resonance (^1H and ^{13}C) spectroscopies. Purity was >98%. A detailed description of the synthesis and analytical data supporting the chemical structure of compounds used here will be published elsewhere. Drugs were prepared as 50 mM stock solutions in DMSO and stored at −26°C. Drugs were added to cells in 1 μl of DMSO/ml. No endotoxin could be detected into these solutions using the *Limulus* amebocyte lysate (Bio-Whittaker, Cambrex, Walkersville, MD; lowest limit of detection 0.06 endotoxin units/ml).

Measurement of drug lipophilicity. Lipophilicity was estimated as described by Zamora et al. (22) with minor modifications. Briefly, HPLC-grade 1-octanol (Sigma) was presaturated with 0.1 M PBS, pH 7.2, and conversely, PBS was presaturated with 1-octanol. Drugs were then dissolved in PBS at a final concentration of 1×10^{-4} M, an equal volume of 1-octanol was added, and the tubes were continuously inverted for 15 min. Drug concentrations in the aqueous and octanol phases were assessed by measuring the absorbance at the most convenient wavelength, as determined from the UV spectra. The partition coefficient D was calculated by dividing the absorbance in 1-octanol by the absorbance in PBS. $Log_{10}D$ was used as a measure of lipophilicity (Fig. 2).

Cells. Alveolar macrophages, obtained by washing the airways of adult rabbits with 145 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 2 mM HEPES, 6 mM glucose, and 0.2 mM EGTA, pH 7.4, were washed two times with Ringer buffer (145 mM NaCl, 5 mM KCl, 2 mM Na_2HPO_4, 1 mM $MgCl_2$, 2 mM HEPES, 10 mM glucose, pH 7.4), suspended in Ringer buffer plus 1 mg/ml of BSA (RBA), and used immediately. The cells were >90% macrophages (May-Grunwald-Giemsa staining) and 95 ± 1% viable (means ± SE, n = 47) as determined by trypan blue exclusion.

Effect of drugs on trypan blue exclusion and on release of lactate dehydrogenase by alveolar macrophages. Macrophages (10^6 cells in 1 ml of RBA) were incubated for 1 h at 37°C with amiodarone or amiodarone analogs, added in 1 μl of DMSO. At the end, a small aliquot was used to study trypan blue exclusion, whereas the remaining cells were sedi-

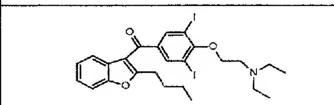
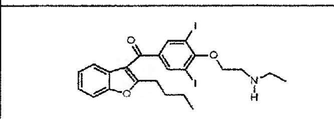
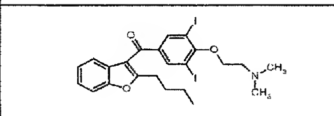
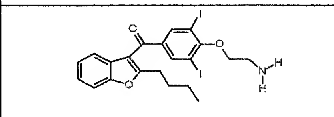
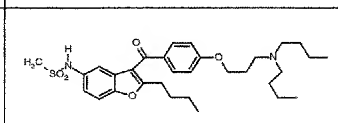
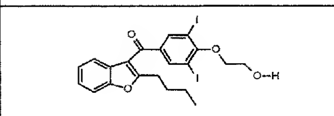
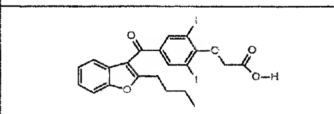
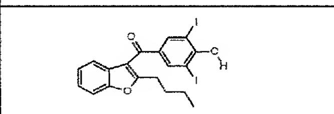
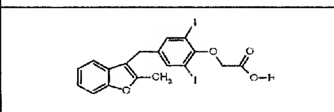
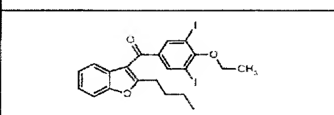
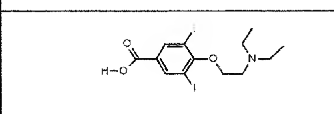
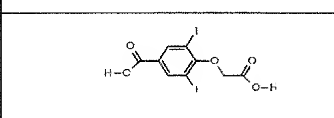
Compound	Chemical Structure	log D
Amiodarone		**3.51**
MDEA		**2.89**
Me-AMI		**2.82**
DDEA		**2.58**
Dronedarone		**2.58**
B2-O-EtOH		**2.44**
B2-O-AcOH		**2.40**
B2		**2.39**
KB130015		**2.27**
B2-O-Et		**2.21**
D2		**- 0.44**
DIB-O-A		**- 1.45**

Fig. 2. Amiodarone analogs. Log D = Log_{10} (absorbance of 1-octanol phase/absorbance of water phase), average of 3 determinations. MDEA, mono-*N*-desethylamiodarone; DDEA, di-*N*-desethylamiodarone. See MATERIALS AND METHODS for description of abbreviated compounds.

mented at 3,000 rpm, and lactate dehydrogenase (LDH) released in 50 μl of the supernatant was measured with a commercial kit (CytoTox 96, Promega). LDH released is presented as % of the LDH liberated from the same cells in the presence of 0.9% Triton X-100 and is compared with LDH released from cells exposed to plain DMSO (control macrophages). Under the present conditions, control macrophages released 2.4 ± 0.7% of total LDH per hour (means ± SE), $n = 6$.

Effect of amiodarone and amiodarone analogs on alveolar macrophage morphology. Macrophages in RBA plus 50 units/ml of penicillin and 50 μg/ml of streptomycin were allowed to adhere for 2 h to six-well Falcon plates (Becton Dickinson Labware Europe, Meylan, France; 2×10^6 cells/plate) and were then cultured for 24 h at 37°C, 5% CO_2, in the presence of DMSO (1 μl/ml, control cells), 10 μM amiodarone, or 10 μM amiodarone analogs. At the end, adhering cells were washed with Tyrode buffer, pH 7.3, scraped, and centrifuged at 10,000 *g*. The resulting pellets were fixed overnight with 2.5% glutaraldehyde in Tyrode buffer, postfixed for 2 h in 1% osmium tetroxide, dehydrated, and embedded in Spurr resin. Semithin sections obtained through the whole thickness of the pellets were stained with toluidine blue and observed with a Zeiss Axiophot light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Jeol 1200 EXGII electron microscope.

Morphometry was performed on 10 electron micrographs of each experimental condition randomly taken at ×4,000 magnification and then photographically enlarged to ×10,000 magnification to appreciate the details of at least 2–3 cells/micrograph. A total number of 25 cells in each experimental condition were counted. By means of a ruler inserted within an optical magnifier, we measured the surface area covered by the whole cell and by vacuoles or cytoplasmic inclusions (i.e., electron-dense structures containing amorphous and/or multilamellar membranes, not surrounded by any type of membrane). All organelles that were detectable in the section of each of the randomly selected cells were analyzed. Results are expressed as % of the surface area covered by different organelles in relation to the surface area covered by the whole cytoplasm.

Preparation and labeling of SP-A. SP-A was isolated from the surfactant obtained from a patient with alveolar proteinosis and labeled with sodium ^{125}I as described (4). ^{125}I-SP-A had a specific activity of 400–600 cpm/ng, migrated as expected during polyacrylamide gel electrophoresis, and was >99% precipitable with 20% cold TCA. ^{125}I-SP-A was stored at 4°C and used within a month.

Effect of amiodarone and amiodarone analogs on the degradation of ^{125}I-SP-A by alveolar macrophages. Alveolar macrophages (10^6 cells in 1 ml of RBA) were incubated for 1 h at 37°C in the presence of different drugs added in 1 μl of DMSO (final drug concentration 0–50 μM). One microgram of ^{125}I-SP-A was then added, and the incubation was continued for one further hour. At the end, the radioactivity soluble in 20% cold TCA was measured in medium plus cells. Degradation of SP-A is presented as % of the degradation of SP-A measured in control cells. Under the present conditions, control cells degraded 46 ± 7 ng of SP-A/10^6 cells/hour (means ± SE, $n = 30$).

Effect of amiodarone, dronedarone, and KB-130015 on the clearance of ^{125}I-SP-A administered into trachea. Sedimentable surfactant (*fraction B* of Ref. 3) freshly obtained from 3-day-old rabbits was suspended in saline, mixed with ^{125}I-SP-A, and then combined with amiodarone, dronedarone, KB-130015, or plain DMSO to make four different mixtures. Two hundred microliters of each mixture contained: 1 μg of ^{125}I-SP-A, 5% of the alveolar pool of phospholipids normally found in 3-day-old rabbits, 250 nmol of one of the drugs in 5 μl of DMSO, or 5 μl of plain DMSO (controls). Surfactant was added to the mixtures to prevent nonspecific binding of SP-A to vials or tubing.

Two hundred microliters of the mixtures were instilled to 3-day-old rabbits (10 rabbits per mixture) by puncturing the trachea after exposing it under local anesthesia. The animals were killed after 3 h with an excess of pentobarbital sodium in the peritoneum, and the airways were lavaged four times with 4–5 ml of 0.9% normal saline. The lavage fluid was used to count cells, to estimate cell viability by trypan blue exclusion, and to measure total and TCA-soluble radioactivity. An aliquot of it was centrifuged for 5 min at 500 *g*, and the supernatant was used to measure proteins and LDH. The washed lungs were homogenized in cold saline, and the radioactivity of the homogenate, total and TCA-soluble, was measured. The radioactivity recovered from lavage fluid and homogenate was expressed as % of the dose instilled.

The animals were killed 3 h after instillation because, after this time interval, administered SP-A is evenly distributed between alveoli and parenchyma and the lung retains enough radioactivity for counting (4). The dose of 250 nmol was chosen because we previously found that 250 nmol of amiodarone instilled into trachea interfere with the clearance of SP-A without clear damage to the lungs (4).

Statistical analysis. Data are expressed as means ± SE. Differences between groups were analyzed by ANOVA using the Student-Newman-Keuls test as the post hoc test for data normally distributed and Dunn's test for data not normally distributed. The level of significance accepted was 5%.

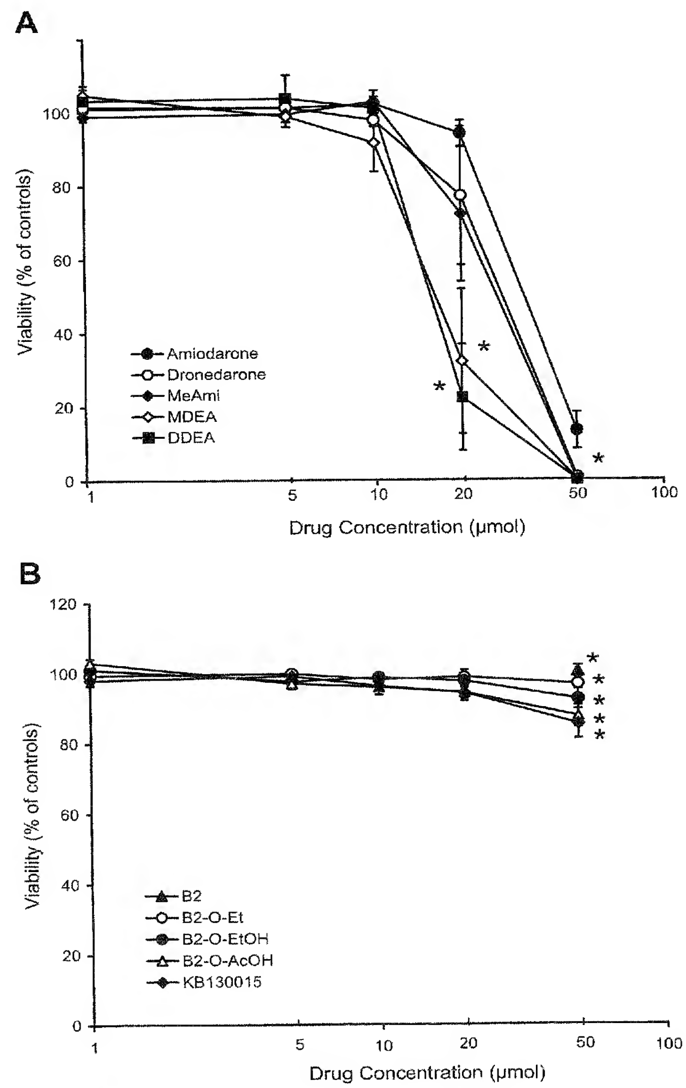


Fig. 3. Effect of analogs of amiodarone on the viability of alveolar macrophages evaluated by trypan blue exclusion. *A*: amiodarone analogs containing an amine function. *B*: other analogs. Data are means ± SE, $n = 4$–6. *Different from amiodarone at the same concentration by ANOVA.

RESULTS

Signs of cell damage. Amiodarone had no effect on trypan blue exclusion up to a concentration of 20 μM. MDEA and DDEA were more powerful than amiodarone, exhibiting a significant inhibition at a concentration of 20 μM (Fig. 3). Dronedarone and MeAMI had an effect similar to that of amiodarone. B2, B2-O-Et, B2-O-EtOH, B2-O-AcOH, and KB-130015 had no effect on trypan blue exclusion up to a concentration of 50 μM (Fig. 3). Analogs lacking the benzofuran group, like the hydrosoluble compounds D2 and DIB-O-A, had no effect on trypan blue exclusion up to a concentration of 50 μM (not shown).

Thus, considering amiodarone metabolites, it appears that MDEA and DDEA are more toxic than amiodarone, whereas B2-O-EtOH has no effect on trypan blue exclusion. Considering all substances studied, it appears that only analogs with an amine function and ranking at the top of the hydrophobicity scale (MDEA, DDEA, MeAMI, and dronedarone) interfere with trypan blue exclusion (Figs. 2 and 3). The finding that the hydrophilic analog D2, which lacks the benzofuran moiety but retains a complete diethylaminoethoxy group, had no effect on trypan blue exclusion, suggests that a certain degree of hydrophobicity is required to affect trypan blue exclusion.

Incubation with 1–50 μM amiodarone had a modest effect on the release of LDH by alveolar macrophages, whereas MDEA, DDEA, and dronedarone induced a dramatic increase (Fig. 4). MeAMI also increased the release of LDH, but the change was not significant (Fig. 4). B2, B2-O-Et, B2-O-EtOH, B2-O-AcOH, and KB-130015 did not increase the release of LDH by alveolar macrophages (Fig. 4). These results reinforce the concept that *1*) amiodarone metabolism generates derivatives with diverse toxicity toward alveolar macrophages, some being more (MDEA and DDEA) and some being less (B2-O-EtOH) toxic than the parent compound and *2*) dronedarone is more, and KB-130015 is less, toxic than amiodarone toward alveolar macrophages. Furthermore, because the uptake of trypan blue and the release of LDH are both due to damage to the plasma membrane (15), it appears that the uptake of trypan blue is a more sensitive index of damage.

Macrophage morphology. As expected (4), exposure to amiodarone had profound effects on macrophages by increasing four times the area covered by inclusion bodies and by >27 times the area covered by vacuoles (Figs. 5–7).

Considering the formation of inclusion bodies, MDEA and dronedarone increased significantly the surface area covered by inclusion bodies (Figs. 5–7). DDEA and B2-O-EtOH did not induce significant changes with respect to control cells, but B2-O-EtOH caused the formation of less inclusion bodies than DDEA. (Figs. 5–7). Thus, amiodarone, MDEA, DDEA, and B2-O-EtOH have a progressively smaller ability to induce the formation of inclusion bodies.

Considering the formation of vacuoles, dronedarone had the strongest effect among the substances tested, increasing the surface area by >37 times. Besides dronedarone, amiodarone metabolites also had a significant effect on the formation of vacuoles but showed dramatic differences in potency. In fact, the ability to induce the formation of vesicles appears to rank in the following order: amiodarone > MDEA ≫ DDEA > B2-O-EtOH (all significantly different from each other by ANOVA) (Fig. 7). Interestingly, some analogs that had no effect on trypan blue exclusion and release of LDH, like B2-O-Et and KB-130015, caused a small but significant increase of the area covered by vesicles (Fig. 7).

Degradation of SP-A by alveolar macrophages. Amiodarone inhibited the degradation of SP-A by alveolar macrophages, the effect starting to be significant at a concentration of 10 μM ($P < 0.05$, ANOVA) (Fig. 8). MDEA, DDEA, MeAMI, and dronedarone inhibited the degradation of SP-A to the same extent of amiodarone (Fig. 8). B2-O-EtOH, B2-O-AcOH, and KB-130015 also inhibited the degradation of SP-A but did so to a lesser extent and without producing the clear sigmoid

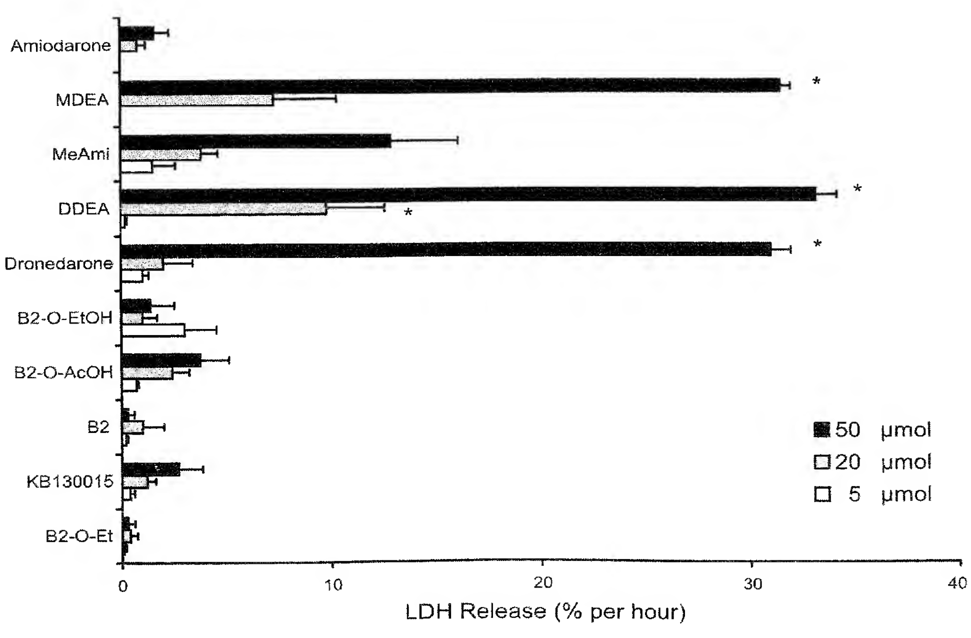


Fig. 4. Effect of analogs of amiodarone on release of lactate dehydrogenase (LDH) by alveolar macrophages. Analogs are in order of decreasing hydrophobicity from *top* to *bottom*. Data (means ± SE) represent % of cell LDH released per hour, after subtraction of LDH released by control cells. $N = 6$. *Different from amiodarone at the same concentration by ANOVA ($P < 0.05$).

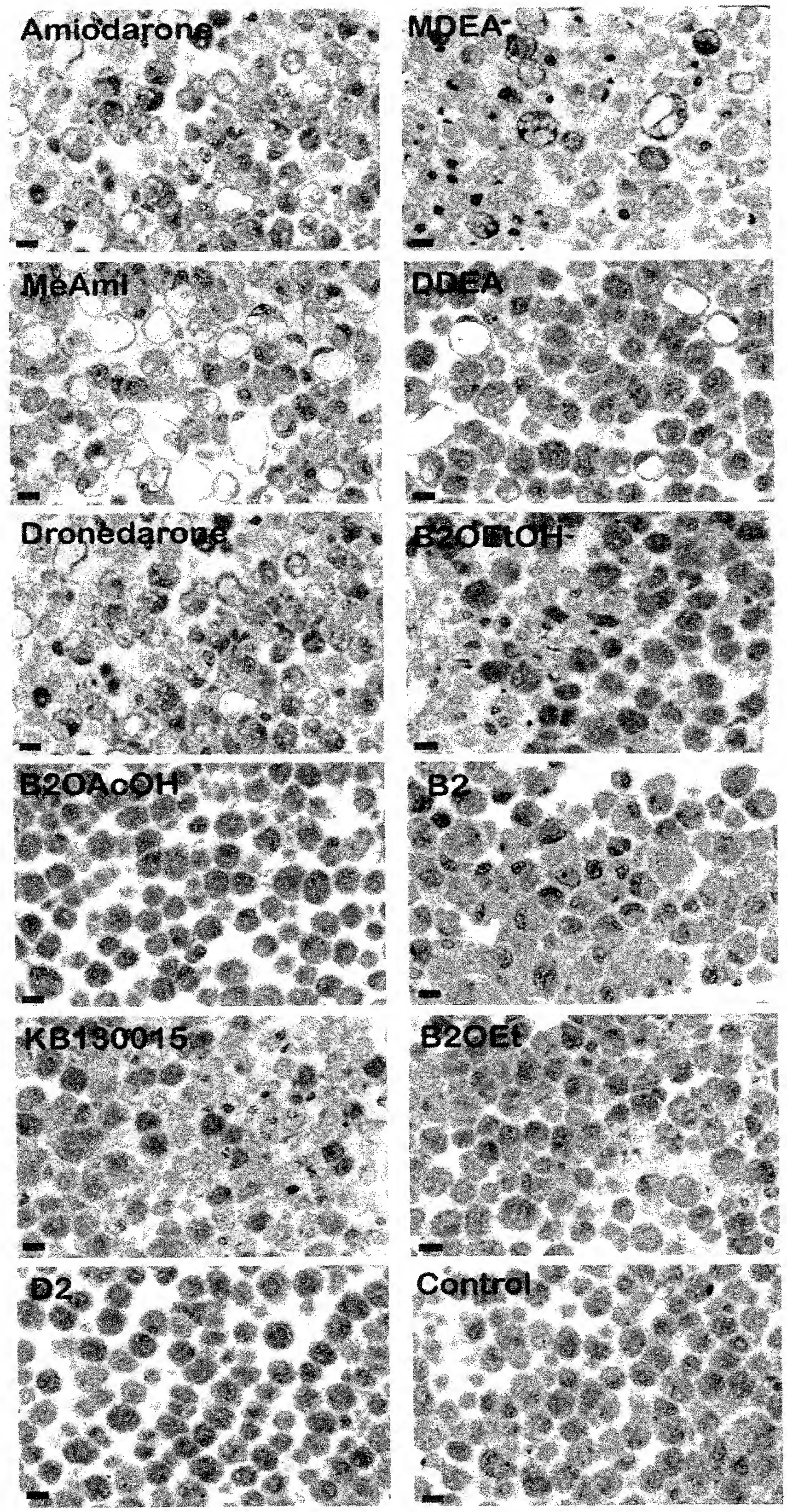


Fig. 5. Effect of amiodarone metabolites and analogs on the morphology of alveolar macrophages by light microscopy. Bars = 10 μm.

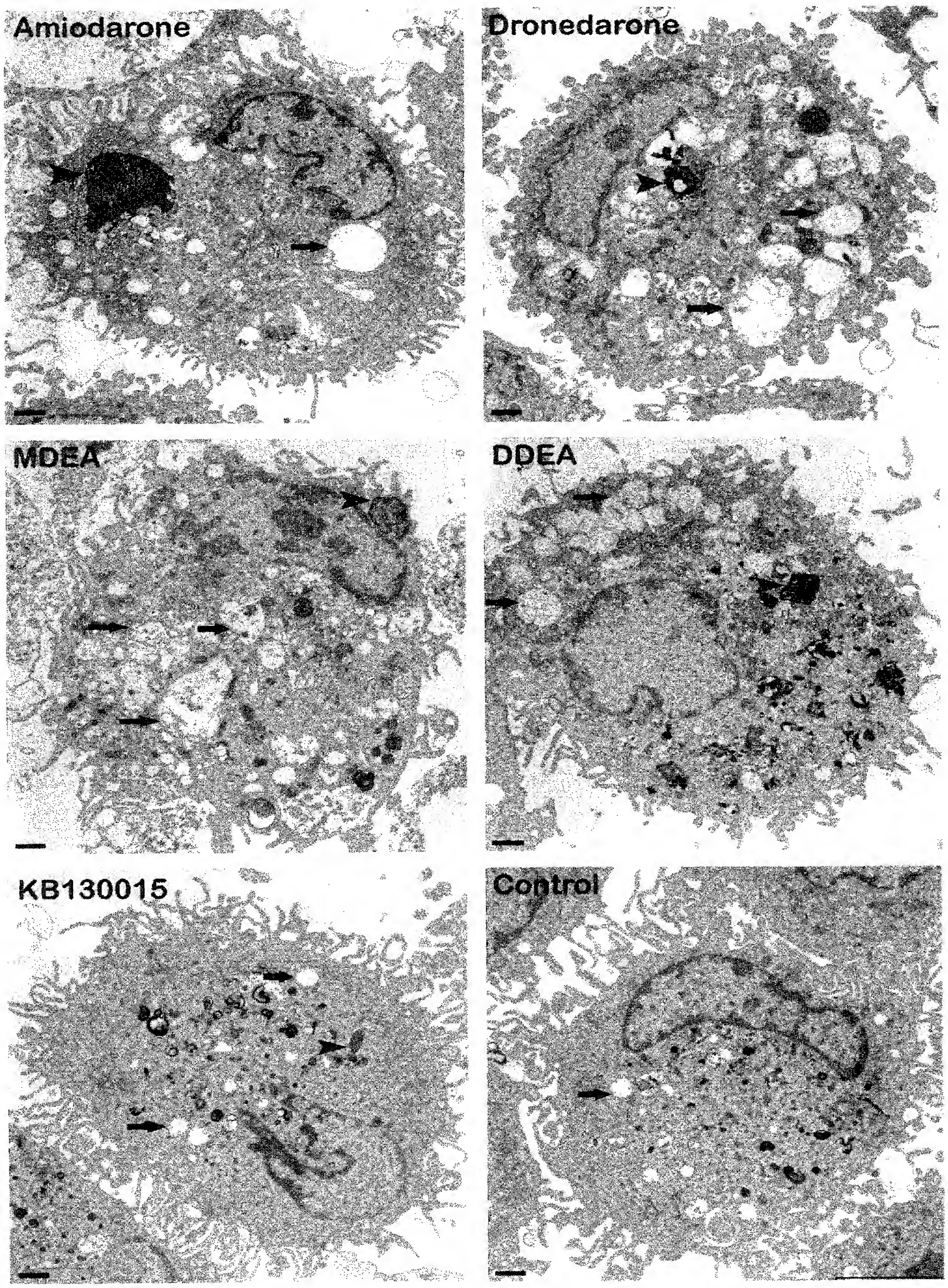


Fig. 6. Effect of amiodarone metabolites and analogs on the morphology of alveolar macrophages by transmission electron microscopy. Arrowheads: inclusion body; arrows: vacuole. Bars = 1 μm.

curve characteristic of the inhibition due to amiodarone (Fig. 8). B2, B2-O-Et, D2, and DIB-O-A had no effect on the degradation of SP-A up to a concentration of 50 μM (Fig. 8, DIB-O-A not shown). Thus it appears that analogs containing an amine function and ranking at the top of the hydrophobicity scale (MDEA, DDEA, MeAMI, and dronedarone) have the greatest ability of inhibiting the degradation of SP-A. Analogs in which the diethylaminoethoxy group was substituted by an ethoxy or an acethoxy group retained some, albeit smaller, inhibitory activity, whereas the substitution of the diethylaminoethoxy group with a hydroxy or an ethyl group abrogated completely the ability to inhibit the degradation of SP-A. These results are compatible with the view that the tertiary nitrogen of amiodarone may play a role in the inhibition of the degradation of SP-A by alveolar macrophages, provided the molecule reaches a certain degree of hydrophobicity. In fact, the hydrophilic compound D2, which contains an intact diethylaminoethoxy group, had no effect on the degradation of SP-A (Fig. 8).

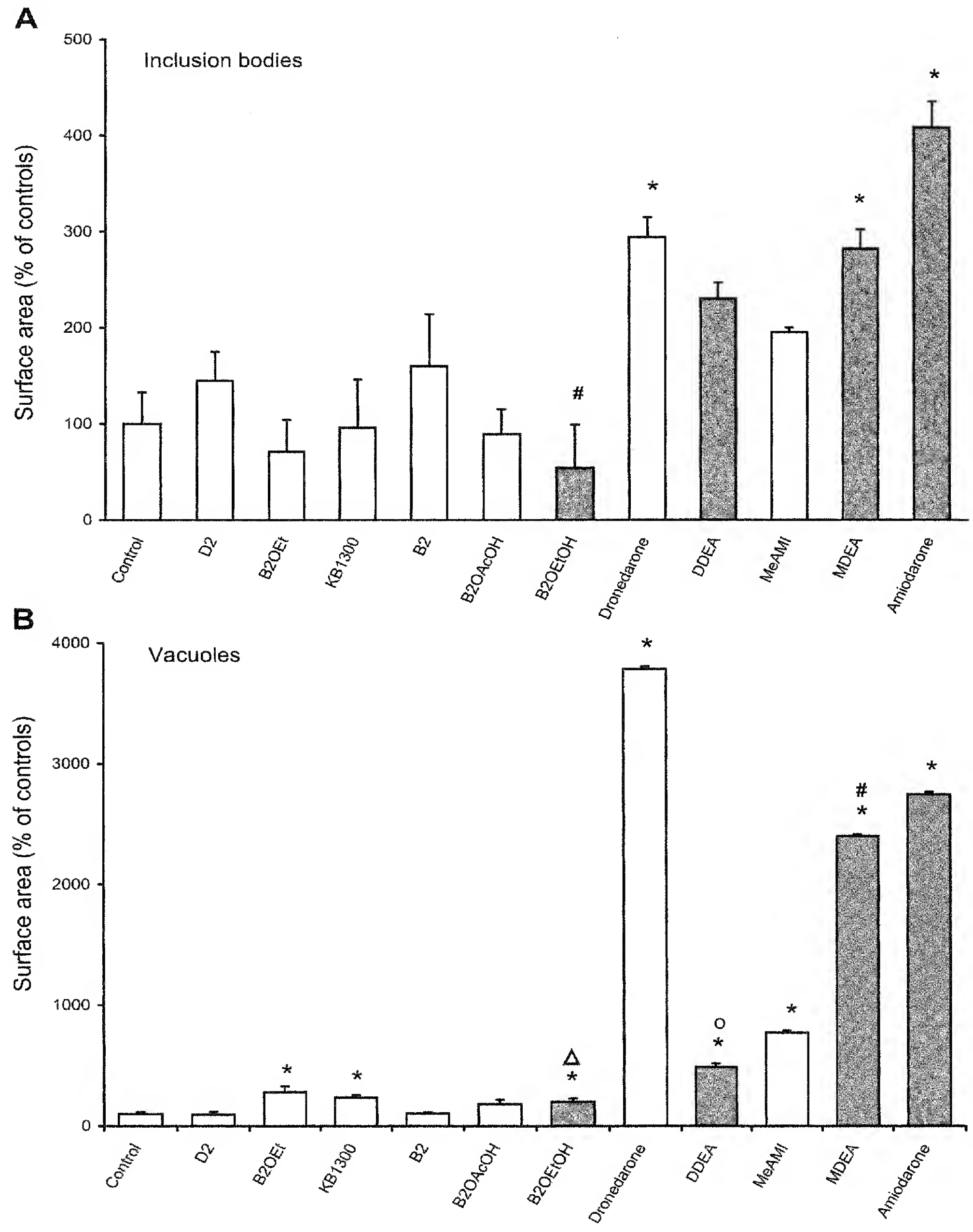


Fig. 7. Effect of amiodarone metabolites and analogs on the surface area covered by inclusion bodies (*A*) and vacuoles (*B*). Compounds arranged in order of increasing hydrophobicity from *left* to *right*. Filled columns represent amiodarone and amiodarone metabolites. Data are means ± SE. In *A*, * different from control, # different from DDEA (ANOVA). In *B*, * different from control, # different from amiodarone, ○, different from MDEA, △, different from DDEA (ANOVA).

Uptake and degradation of SP-A administered through the airways. In control animals, 3 h after the tracheal instillation of ^{125}I-SP-A, the total radioactivity was evenly distributed between airways and lung tissue, and degradation products were present, especially in the airways (Figs. 9 and 10). Amiodarone inhibited the degradation of SP-A after the uptake, with the consequence that less degradation products were formed and nondegraded SP-A accumulated into lung tissue (Figs. 9 and 10). Dronedarone had an effect comparable with that of amiodarone, causing the accumulation of large amounts of nondegraded SP-A into lung tissue (Figs. 9 and 10). KB-130015 had a much smaller effect, causing a small accumulation of nondegraded SP-A in lung tissue (Figs. 9 and 10).

DISCUSSION

MDEA, DDEA, and B2-O-EtOH are metabolites of amiodarone of decreasing hydrophobicity produced by sequential modifications of the diethylaminoethoxy group (Fig. 2). Although the toxicity of MDEA is known in part (5), the effects of DDEA and B2-O-EtOH have never been studied.

We found that the toxicity of MDEA toward alveolar macrophages is greater than that of amiodarone according to trypan blue exclusion and the release of LDH. On the other hand, the toxicity of MDEA is similar to that of amiodarone, considering the effects on the degradation of SP-A and the formation of inclusion bodies, and is smaller than that of amiodarone, if one

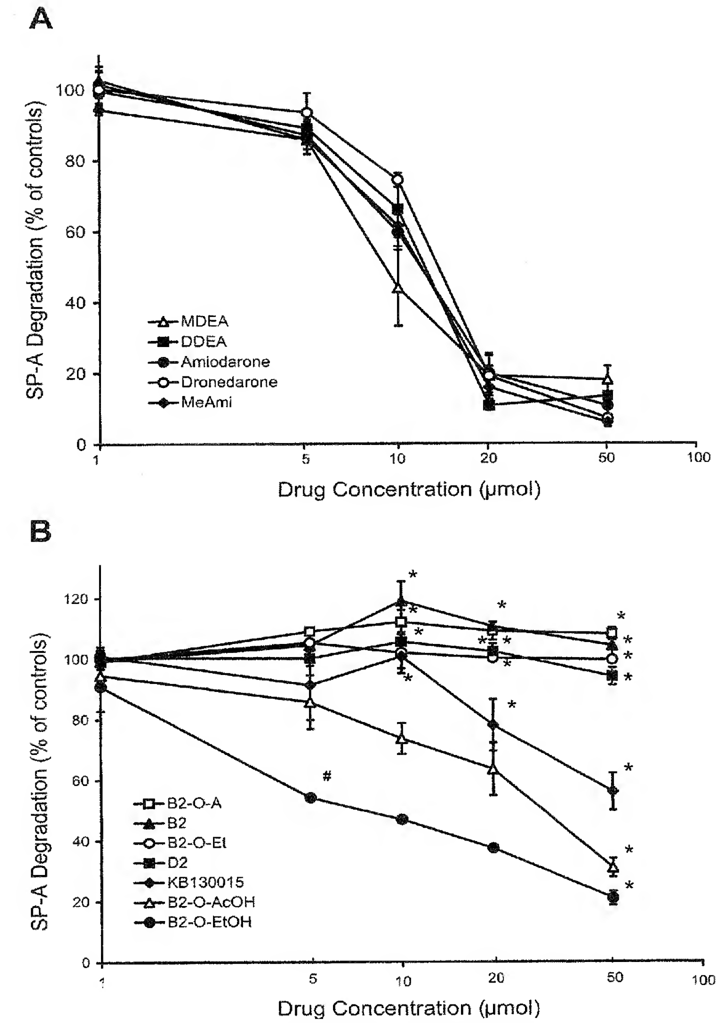


Fig. 8. Effect of analogs of amiodarone on the degradation of surfactant protein A (SP-A) by rabbit alveolar macrophages. *A*: amiodarone metabolites and analogs containing an amine function. *B*: other metabolites and analogs. Data are % of SP-A degraded by control cells (means ± SE); n = 3–7. *Greater, #smaller than amiodarone at same concentration by ANOVA.

looks at the formation of vacuoles. Deethylation of MDEA to DDEA dramatically decreases the ability to cause the formation of vacuoles in the cytoplasm of alveolar macrophages, whereas the rest of the toxicity profile remains unchanged. Deamination of DDEA reduces the toxicity further, since the resulting metabolite (B2-O-EtOH) has no effect on trypan blue exclusion and the release of LDH, has modest effects on morphology, and retains some ability to inhibit the degradation of SP-A. In addition to an improvement of the toxicity profile, the deamination of DDEA could have a further positive effect by influencing pharmacokinetics, since the plasma of patients receiving amiodarone contains measurable amounts of MDEA and DDEA, whereas B2-O-EtOH remains under the limit of detection, possibly because it is quickly eliminated by conjugation (unpublished observations). Thus MDEA, DDEA, and B2-O-EtOH rank in order of decreasing toxicity toward alveolar macrophages, indicating that dealkylation and deamination of the diethylaminoethoxy group are important steps in the

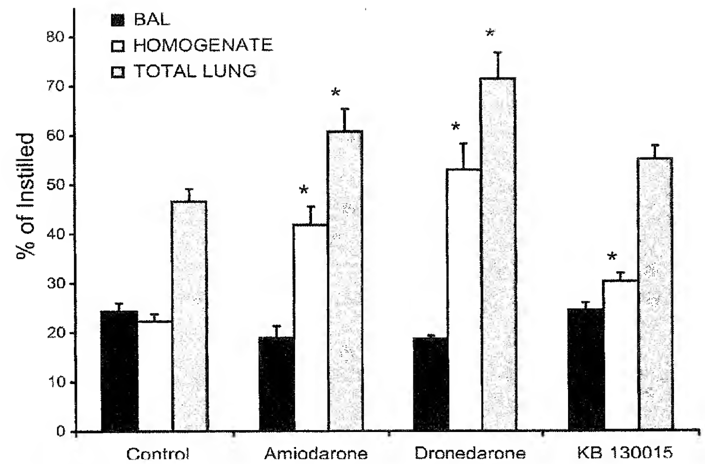


Fig. 9. Effect of amiodarone, dronedarone, and KB-130015 on the clearance of ^{125}I-SP-A administered into trachea. Drugs (250 nmol) were mixed with ^{125}I-SP-A and then instilled into trachea of 3-day-old rabbits that were killed after 3 h. Shown is the distribution of total radioactivity in lung lavage fluid (BAL), lung homogenate, and total lung. Data are means ± SE. N = 14 for amiodarone, 4 for dronedarone, and 10 for KB-130015. *Different from control in the same compartment (ANOVA).

biotransformation of amiodarone. The crucial role of this lateral group in the toxicity toward macrophages also emerges from the study of analogs. In fact, the deletion of the diethylaminoethoxy group of amiodarone generates B2, an analog devoid of all toxic effects considered in this study. Because B2 contains the diiodobenzoyl moiety and the benzofuran moiety with its associated butyl group (Figs. 1 and 2), it appears that these parts of the amiodarone molecule play no direct role in the toxicity toward alveolar macrophages. This finding contrasts sharply with the results of studies exploring the mechanism of amiodarone toxicity toward the liver (19). Those experiments showed unambiguously that the benzofuran moiety is responsible for liver toxicity, suggesting that different

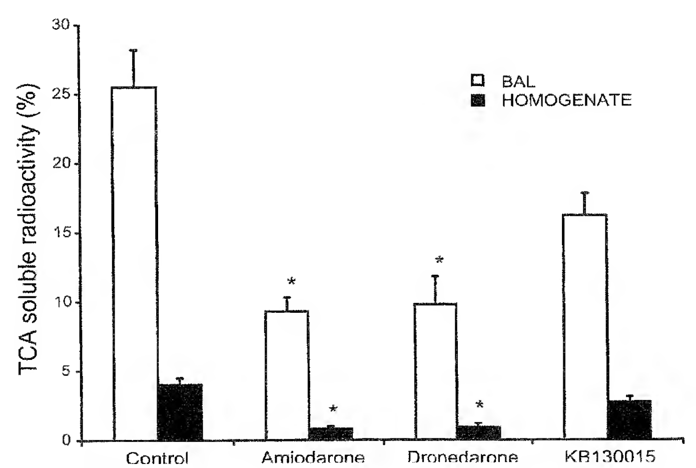


Fig. 10. Effect of amiodarone, dronedarone, and KB-130015 on the distribution of TCA-soluble radioactivity in BAL, lung homogenate, and total lung after administration of ^{125}I-SP-A into trachea. Data are means ± SE. N = 10 for amiodarone, 4 for dronedarone, and 10 for KB-130015. *Different from control in the same compartment (ANOVA).

parts of the amiodarone molecule could be toxic toward specific cell types.

Amiodarone interferes with various cell functions and structures, such as late steps of endocytosis (4), the catabolism of proteins and lipids (4, 14), mitochondrial integrity and energy metabolism (13), calcium homeostasis (8, 12), ion pumps (11), and the distribution of receptors among different cell compartments (6, 20). From the evidence presented here, it appears that amiodarone metabolites may also have a plethora of effects since they impair the integrity of the plasma membrane, interfere with the degradation of SP-A, which normally is taken up by macrophages and degraded into lysosomes (4), and impact on the turnover of cell organelles, as indicated by the accumulation of vesicles and multilamellar inclusion bodies.

The nitrogen of the diethylaminoethoxy group appears to play an important role in toxicity toward alveolar macrophages because analogs and metabolites containing it rank at the top of the toxicity scale (amiodarone, MDEA, DDEA, MeAMI, dronedarone) and because its removal during amiodarone metabolism dramatically decreases adverse effects toward macrophages. The presence of a tertiary nitrogen is typical of lysosomotropic amines that, in their neutral form, enter the lysosomes by diffusion but then, in the acidic lysosomal milieu, become insoluble and accumulate to high levels, causing the osmotic swelling of lysosomes (9). Thus it is tempting to speculate that some of the effects of amiodarone might be due to interference with the lysosomes by this mechanism. It is worth noting, however, that lysosomotropic amines with vacuoligenic ability, like methylamine and chloroquine, work at millimolar concentrations (1), whereas amiodarone and its metabolites work at micromolar concentrations. Furthermore, we find that during amiodarone degradation, the greatest decrease in the vacuoligenic ability is due to deethylation of MDEA rather than to deamination of DDEA (Figs. 5–7). Finally, we show that analogs lacking the amine function retain some ability of generating vacuoles (like B2-O-EtOH, B2-O-Et, and KB-130015) and can still interfere with the degradation of SP-A (B2-O-EtOH, B2-O-AcOH, and KB-130015). Thus the role of the tertiary nitrogen of amiodarone in macrophage toxicity remains to be fully defined.

One aim of this investigation was to analyze the effects on alveolar macrophages of two amiodarone derivatives with proven antiarrhythmic activity, dronedarone and KB-130015, and to compare them with those of amiodarone. Our results indicate that, at equimolar concentrations, dronedarone has a toxicity greater than or equal to that of amiodarone since it inhibits to a greater extent the exclusion of trypan blue, causes a greater release of LDH, and has a higher vacuolating ability than amiodarone. On the other hand, dronedarone affects the clearance of SP-A to the same extent as amiodarone, both in vivo and in vitro. KB-130015 is less toxic than amiodarone toward alveolar macrophages according to all criteria used in this study and presents a toxicity profile similar to that of B2-O-EtOH.

The overall picture that emerges from this study can be summarized as follows: *1*) amiodarone metabolites MDEA, DDEA, and B2-O-EtOH rank in order of decreasing toxicity toward alveolar macrophages, indicating that dealkylation and deamination of the diethylaminoethoxy group are important mechanisms of detoxification; *2*) dronedarone and KB-130015 have respectively greater and smaller toxicity toward alveolar macrophages than amiodarone; and *3*) the benzofuran moiety, which is toxic to liver cells, is not directly toxic toward alveolar macrophages.

It is important to note that these conclusions are based on a limited number of effects observed on isolated cells after incubation with drugs present at concentrations that may or may not be reached in vivo. Furthermore, many of the most important effects of amiodarone, such those on mitochondria, on the traffic of lysosomal enzymes, on calcium homeostasis, and on the regulation of apoptosis (2, 10) were not examined in the present investigation. Finally, it should be considered that effects interpreted here as toxic toward alveolar macrophages, such as the interference with the traffic of vesicles, could represent a desirable end point in other respects, like the recently discovered antifungal activity of amiodarone (12).

ACKNOWLEDGMENTS

We thank Prof. Gaetano Crepaldi for continuous support and encouragement. We also thank Raffaella Marin, Sabrina Pigozzo, Dr. Irene Cortella, Dr. Daniela Conte, and Dr. Beniamino Poggiana for technical help. We thank Dr. Roberto Dragagna from Jacopo Monico SPA (Mestre, Venice, Italy) for performing the endotoxin assay.

GRANTS

This work was supported in part by Fondi 60%, University of Padua (to A. Baritussio and A. Pettenazzo).

REFERENCES

1. **Arai K, Yasuda N, Isohashi F, Okamoto K, and Ohkuma S.** Inhibition of weak-base amine induced lysis of lysosomes by cytosol. *J Biochem* 132: 529–534, 2002.
2. **Bargout R, Jankov A, Dincer E, Wang R, Komodromos T, Ibarra-Sunga O, Filippatos G, and Uhal BD.** Amiodarone induces apoptosis of human and rat alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 278: L1039–L1044, 2001.
3. **Baritussio A, Bellina L, Carraro R, Rossi A, Enzi G, Magoon MW, and Mussini I.** Heterogeneity of alveolar surfactant in the rabbit: composition, morphology and labeling of subfractions isolated by centrifugation of lung lavage. *Eur J Clin Invest* 14: 24–29, 1984.
4. **Baritussio A, Marzini S, Agostini M, Alberti A, Cimenti C, Bruttomesso D, Manzato E, Quaglino D, and Pettenazzo A.** Amiodarone inhibits lung degradation of SP-A and perturbs the distribution of lysosomal enzymes. *Am J Physiol Lung Cell Mol Physiol* 281: L1189–L1199, 2001.
5. **Brien JF, Jimmo S, Brennan FJ, Pichard L, Ford SE, and Armstrong PW.** Distribution of amiodarone and its metabolite, desethylamiodarone, in human tissues. *Can J Physiol Pharmacol* 65: 360–374, 1987.
6. **Budu CE, Balas N, Nawrath H, Wegener JW, and Shainberg A.** Increased cardiac α-1-adrenoreceptor density in rats following treatment with amiodarone. *J Basic Clin Physiol Pharmacol* 12: 33–47, 2001.
7. **Carson B, Singh BN, Temciuc M, Nilsson S, Li YL, Mellin C, and Malm J.** Synthesis of a novel antiarrhythmic compound (KB130015) with improved toxicity profile compared with amiodarone. *J Med Chem* 31: 623–630, 2002.
8. **Courchesne WE and Ozturk S.** Amiodarone induces a caffeine inhibited, MID1-dependent rise in free cytoplasmic calcium in *Saccharomyces cerevisiae*. *Mol Microbiol* 47: 223–234, 2003.
9. **De Duve C, de Barsy T, Poole G, Trouet A, Tulkens P, and van Hoof F.** Lysosomotropic drugs. *Biochem Pharmacol* 23: 2495–2531, 1974.
10. **Di Matola T, D' Ascoli F, Fenzi G, Rossi G, Martino E, Bogazzi F, and Vitale M.** Amiodarone induces cytochrome C release and apoptosis through a iodine-independent mechanism. *J Clin Endocrinol Metab* 85: 4323–4330, 2000.

11. **Futamura Y.** Effect of amiodarone on cytokine release and on enzyme activity of mouse alveolar macrophages, bone marrow macrophages and blood monocytes. *J Toxicol Sci* 21: 125–134, 1996.
12. **Gupta SS, Ton VK, Beaudry V, Rulli S, Cunningham K, and Rao R.** Antifungal activity of amiodarone is mediated by disruption of calcium homeostasis. *J Biol Chem* 31: 28831–28839, 2003.
13. **Ha HR, Stieger B, Grassi G, Altorfer HR, and Follath F.** Structure-effect relationships of amiodarone analogues on the inhibition of thyroxine deiodination. *Eur J Clin Pharmacol* 55: 807–814, 2000.
14. **Hostetler KY, Giordano JR, and Jellison EJ.** In vitro inhibition of lysosomal phospholipase A_1 of rat lung by amiodarone and desethylamiodarone. *Biochim Biophys Acta* 959: 316–321, 1988.
15. **Korzeniewski C and Callewaert DM.** An enzyme-release assay for natural cytotoxicity. *J Immunol Methods* 64: 313–320, 1983.
16. **Naccarelli GV, Wolbrette DL, Khan M, Bhatta L, Hynes J, Samii S, and Luck J.** Old and new antiarrhythmic drugs for converting and maintaining sinus rhythm in atrial fibrillation: comparative efficacy and results of trials. *Am J Cardiol* 20: 15–26, 2003.
17. **Ohyama K, Nakajima M, Suzuki M, Shimada N, Yamazaki H, and Yokoi T.** Inhibitory effects of amiodarone and its N-deethylated metabolite on human cytochrome P450 activities: prediction of in vivo drug interactions. *Br J Clin Pharmacol* 49: 244–253, 2000.
18. **Pollak PT, Bouillon T, and Shafer SL.** Population pharmacokinetics of long-term oral amiodarone therapy. *Clin Pharmacol Ther* 67: 642–652, 2000.
19. **Spaniol M, Bracher R, Ha HR, Follath F, and Krahenbuhl S.** Toxicity of amiodarone and amiodarone analogues on isolated liver mitochondria. *J Hepatol* 35: 628–636, 2001.
20. **Vassy R, Starzec A, Yin Y, Nicolas P, and Perret GY.** Amiodarone has exclusively non-genomic action on cardiac β-adrenoreceptors. *Eur J Pharmacol* 24: 227–232, 2000.
21. **Verduyn SC, Vos MA, Leunissen HD, van Opstal JM, and Wellens HJ.** Evaluation of the acute electrophysiologic effects of intravenous dronedarone, an amiodarone-like agent, with special emphasis on ventricular repolarization and acquired torsade de pointes arrhythmias. *J Cardiovasc Pharmacol* 33: 212–222, 1999.
22. **Zamora JM, Pearce L, and Beck WT.** Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukaemic cells. *Mol Pharmacol* 33: 454–462, 1988.